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Mitochondrial mRNA Translation is Required for Maintenance of Oxidative Capacity

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Mitochondrial mRNA Translation is Required
for Maintenance of Oxidative Capacity

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Abstract

Oxidative metabolism is required to produce adequate energy to sustain human life. A primary example of deteriorating oxidative capacity is seen in the cardiac musculature during chronic heart failure. This suggests that by improving oxidative potential, chronic heart disease could be mitigated and one approach to accomplish this may be through targeting the mt-mRNA translation system. **Purpose:** This investigation's purpose was to characterize disruptions in mt-mRNA translation machinery in multiple forms of cardiomyopathy and to determine if mitochondrial mRNA translation initiation factor (mtIF2) is necessary to maintain oxidative capacity in cardiomyocytes. **Methods** Using a combination of animal and cell culture experiments, we first analyzed the oxidative detriments of the myocardium using an LLC tumor implantation model and followed by assessing how antioxidant protection against LLC-conditioned culture media. Additionally, we used a model of concurrent aging and high fat-diet induced cardiac hypertrophy. After identifying mtIF2 as a potential contributor to oxidative detriments in the heart, we used genetic alterations of H9c2 cardiomyocytes to characterize its necessity. Analyses performed in animal and culture experiments include optical metabolic imaging, immunoblot of mitochondrial quality controllers, bioenergetics flux analysis and hypoxic resistance, **Results:** LLC-implanted animal hearts demonstrated ~15% lower optical redox ratio (FAD/FAD+NADH), a marker for greater glycolytic reliance compared to controls. mt-mRNA translation machinery was unchanged between groups relative to amount of mitochondria. Mitochondrial DNA-encoded CytB was ~30% lower in LLC hearts suggesting impairments in outcomes of mitochondrial mRNA translation. Aged mouse hearts were larger and contained less mtIF2 protein alongside reduced content of CytB. Reducing the content of mtIF2 is associated with reduced oxidative characteristics such as OXPHOS complex I and IV

content, optical redox ratio, oxygen consumption, and viability following hypoxia. **Conclusion:** In conclusion, the research investigations presented within this dissertation are the first to establish mitochondrial mRNA translation as a process that is dysregulated during cardiometablic disease and as a potential therapeutic target to enhance oxidative characteristics of the myocardium. mtIF2 presents as a key regulator for the process of mt-mRNA translation and is necessary for maintain oxidative capacity in cardiac muscle.

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This work would not have been possible without the direction and guidance of the educators that challenged me to become better than they were, and the family that actually understood how far we could go.

Dedication

Dedicated to the two best examples of what I want to become: Wilmer Loyd Powell (Auguest 23rd, 1927 – October 3rd, 2008) and Jonathan Stuart Lee, I (June 12th, 1914 – August 30th, 2006).

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List of Abbreviations:

aa-tRNA aminoacylated-transfer RNA

AHA American Heart Association

AMPK AMP-activated protein kinase

AP Angina Pectoralis

ATP Adenosine triphosphate

BMI Body mass index

BNIP3 Bcl2 interacting protein

CAD Coronary Artery disease

CDC Center for Disease Control and Prevention

CHD Coronary heart disease

COX Cytochrome C oxidase proteins

CPET Cardiopulmonary exercise testing

CVD Cardiovascular disease

DASH Dietary approach to stop hypertension

ERR Estrogen related receptor

ETC Electron transport chain

FeS Iron/sulfur clusters

FFA Free fatty acyls

FMN Flavin mononucleotide

GTP Guanine triphosphate

HBP High blood pressure or hypertension

HF Heart Failure

HSP Heavy strand promoter

IMM Inner mitochondrial membrane

LLC Lewis Lung Carcinoma

LVEF Left ventricular ejection fraction

LSP Light strand promoter

LSU Large ribosomal subunit

MI Myocardial Infarction

mtDNA mitochondrial DNA

MTEF mitochondrial translation elongation factor

MTERF mitochondrial transcription termination factor

MTIF mitochondrial translation initiation factor

mt-mRNA mitochondria-encoded messenger RNA

ND NADH dehydrogenase proteins

nDNA nuclear DNA

NIX BNIP3-like protein

NRF Nuclear receptor factor

OMM Outer mitochondrial membrane

OXPHOS Oxidative Phosphorylation process

PAD Pulmonary Artery disease

PDH Pyruvate dehydrogenase

PPAR Peroxisome proliferator activation receptor

Q Ubiquinone

ROS Reactive oxygen species

SDH Succinate dehydrogenase

SOD Superoxide dismutase

SR Sarcoplasmic Reticulum

SSU Small ribosomal subunit

TCA Tricarboxcylic acid cycle

TFAM Transcription factor A of the mitochondrion

TFB2M Transcription factor B-2 of the mitochondrion

TSS Transcription start site.

List of Submitted Papers

Chapter 3 - Cancer-induced Cardiac Atrophy Adversely Affects Myocardial Redox State and Mitochondrial Oxidative Capacity (Under Review – *Free Radical Biology and Medicine*)

Chapter 4 - Mitochondrial Translation Initiation Factor 2 is Necessary for Cardiac Oxidative Capacity as Evident during Age-induced Cardiac Hypertrophy (In submission – *American Journal of Physiology – Heart and Circulatory Physiology*)

Chapter 1 – Introduction

Significance

Total CVD is the most common cause of death worldwide (>31% of all deaths)[1]. This includes many of the costliest diseases in the developed world such as heart failure, myocardial infarction, coronary artery disease, and stroke[1,2]. Over a century of research has contributed to the fields of heart diseases, biochemistry and oxidative metabolism, including the discovery and characterization of the organelle responsible for energy production within the heart – the mitochondrion[3,4]. Only recently have researchers been able to show the interconnectedness between energy production allowing the heart to pump blood and how it can lead to heart disease[5]. Heart disease, in turn, leads to degeneration of mitochondria through free radicals further damaging the energy production machinery and allowing the disease to progress[6].

Heart disease – Cardiomyopathy is clinically recognized by symptoms of congestive heart failure, reduced left ventricular ejection fraction, enlarged ventricular chamber walls, dilated left ventricle, or poor cardiopulmonary exercise test performance[1]. These symptoms occur when the contractile muscle cells of the heart become stiff as a result of buildup of non-contractile tissue such as scar tissue and collagen. When the heart develops this buildup of fibrotic tissue, it is less efficient at pumping blood throughout the body and requires greater force to produce the same cardiac output. Furthermore, in order to compensate for this inefficiency, the cardiac cells contract more frequently and thus consume and metabolize more energy. Metabolic pressure alters the molecular behaviors of cardiac energy metabolism.

Perinatal cardiomyocytes demonstrate a surprising reliance on glycolytic metabolism. At birth, a shift towards oxidative metabolism of fatty acids occurs concomitant with expansion of the number of mitochondria within the cardiomyocyte[7]. During healthy, adult myocardial

metabolism, up to 90% of the energy for contraction is generated through β-oxidation of fatty acids[8]. As heart disease progresses, the cardiomyocyte cannot provide the necessary energy through fatty acids and begins to rely on glycolytic metabolism[9]. This temporally shifts the cell back towards this perinatal state[10]. Current research attempts to mitigate heart disease progression by improving the reliance on fatty acid substrates for energy metabolism and this seems to contribute to decreased mortality. The location of fatty acid metabolism – the mitochondrion – seems to play a key role in this metabolic shift. Importantly, the mitochondria also generate many of the signals for the cell to begin apoptosis or shift metabolic states. For this reason, understanding how the mitochondria function during heart disease progression can improve understanding for approaches to treat the disease[9].

Mitochondria – Greater than 30% of the volume of cardiomyocytes is attributed to mitochondria. These organelles turnover roughly the body's weight in ATP each day through oxidative metabolism[11]. This process is highly efficient and capable of operating *in vivo* at 80-90% of the enzymatically defined maximal capacity[11,12]. Within the mitochondria are necessary enzymes for the processes of β-oxidation, the TCA cycle, redox handling, mtDNA, proteins to allow mtDNA expression as well as numerous other regulatory and functional processes. Evolution has maintained the necessity to use the mitochondria to produce energy efficiently but has done so by transferring >99.5% of the mitochondrial genome to nuclear DNA[13–15]. The fact that mammalian mitochondria still contain 13 protein-encoding genes that are vital for oxidative metabolism indicates a biological necessity to maintain a separate and energetically expensive set of mtDNA along with hundreds of associated proteins. Each of the proteins encoded by mtDNA are core subunits of the oxidative phosphorylation system complexes[16]. Mutations in these genes are associated with system-wide diseases that stem from poor energy production[15].

Unfortunately, mtDNA is highly susceptible to mutation because of 1) lack of dedicated mtDNA repair machinery, 2) no sexual recombination upon replication, 3) an incomplete mitochondrial degradation that causes potential replication of damaged mtDNA and 4) proximity to the electron transfer machinery[17]. The latter is of importance because of the potential for improper electron transfer to generate mutagenic free radicals.

Electron transport and ROS – Peter Mitchell's chemiosmotic theory explains the movement of electrons through the ETS on the inner mitochondrial membrane to generate an electrochemical gradient, $\Delta\psi_m$, used to produce ATP[18,19]. The electron transfer proteins (Complexes I-IV) are highly complex and utilize an elaborate series of oxidation/reduction reactions to transfer electrons through a number of electron carriers[20]. These electron carriers assist in generation of $\Delta\psi_m$ and allow complex V (ATP synthase) to use this potential to generate ATP[21,22]. This electron transport process is susceptible to producing harmful byproducts in the form of superoxide O_2^- which rapidly generates other forms of reactive oxygen species (ROS)[15,23]. Under healthy metabolic conditions, the cell (and mitochondria) are able to neutralize these free radicals by using a combination of dismutase and catalase enzymes turning the superoxide into harmless water[24]. However, under conditions of impaired metabolism or oxidative stress, the ETS can generate enormous amounts of superoxide which exceed the capabilities of the ROS mitigation system of the cell. These free radicals induce serious damage to cellular components including lipid peroxidation of biomembranes, protein oxidation forming aggregates, advanced glycation end products, cleaved RNAs and greater than 25 known alterations to DNA[24–26]. The mutations in DNA include base modifications that prevent transcription or replication, alterations that generate SNPs, and single strand breaks from deoxyribose scission[26–32]. Because of the limited machinery for mtDNA repair, these mutations

accumulate and cause severe detriments to oxidative capabilities leading to even further ROS production and a self-propagating cycle ensues. Many of the ETS core proteins are encoded by mtDNA and synthesized within the matrix by mt-mRNA translation machinery[33].

mt-mRNA translation – Expression of mtDNA-encoded genes requires a discreet set of translation machinery within the mitochondrial matrix including mitoribosomes, mt-tRNAs, and a specialized set of proteins controlling the translation processes[33–35]. These proteins include initiation factors (mtIFs), elongation factors (mtEFs), and translation activators that govern each step of the mt-mRNA translation process. Because translation initiation is a committal step and polypeptide elongation is extremely energetically expensive (2-4 ATP/peptide), these translation factors are important controllers of the processes[36]. Specifically, mammalian mtIF2 has taken on the historical functions of both bacterial and eukaryotic IFs-1and -2 (binding of 28S mitoribosomal SSU and GTP-dependent association of fMet-tRNA with SSU P-site, respectively)[37]. mtIF3 contains a tether-like linker region with binding activity of both mitoribosomal subunits allowing formation of the complete initiation complex[38]. mtEFTu provides GTPase activity for peptide bond formation between multiple aa-tRNAs, and TACO is a translational activator which assists in colocalization of each piece of the translation system to the mitochondrial membrane where the protein will be inserted[39,40]. The tightly regulated process of mt-mRNA translation requires adequate maintenance of this machinery and is subject to alterations during metabolic stressors such as cardiomyopathies[41].

Previous Research – Decades of investigation have identified metabolic dysfunctions including mitochondrial detriments in the pathology of heart disease[9,42,43]. A transition away from fatty acid metabolism and increased oxidative demand to maintain cardiac output result in stress on key ETC complexes, greater reduction of electron carriers, and thus elevated rates of

ROS production[44–4748]. Previous research demonstrates that elevated ROS production enhances mtDNA mutations, causes early onset heart failure, and decreases lifespan[17,26,49–51]. However, the opposite holds true that enhancing ROS mitigation can limit mtDNA mutations and extend lifespan in rodents[52]. Mutations in key protein controllers of mt-mRNA translation result in altered ETC capacity and greater ROS production[15]. Furthermore, my previous research suggests mt-mRNA translation machinery as being sensitive to change by metabolic stimuli such as obesity or exercise (Figure 1)[41,53]. This suggests that mt-mRNA translation machinery may be dysregulated during cardiomyopathy. One approach to control heart disease may be to limit oxidative stress in the mitochondria by controlling the mt-mRNA translation/electron transport complex/ROS production circuit[6]. In order to assess this approach to combat heart disease, we must first identify how mt-mRNA translation machinery affects oxidative capacity and ROS production and if the **mt-mRNA translation machinery is required for oxidative metabolism and is disrupted in myocardial pathologies.** Experiments to test this central hypothesis will be designed according to these specific aims.

Innovation

Cancer is one of the deadliest diseases in the world second only to heart disease[2]. These experiments will examine an untouched area controlling oxidative metabolism – mt-mRNA translation. We will experimentally define the role of mt-mRNA translation in maintaining oxidative capacity and mitigating ROS production. In doing so, we will assess oxidative characteristics in two unique and understudied models of heart disease: cardiac hypertrophy resulting from the combination of aging-induced sarcopenia coupled with high fat diet-induced obesity as well as cardiac atrophy seen commonly as a side effect of cancer wasting syndromes. The approaches that we will use to assess oxidative capacity and redox states of the

cardiomyocytes are highly innovative because they have not been used previously, in this combination, to assess the oxidative characteristics of cardiac cells during these chronic disease states and the most appropriate in testing the central hypothesis.

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Chapter 2 - Review of Literature

Total CVD is the most common cause of death worldwide (>31% of all deaths). This includes many of the costliest diseases in the developed world such as HF, MI, CAD, and stroke. Over a century of research has contributed to the field of heart diseases, biochemistry and oxidative metabolism, followed by the discovery and characterization of the organelles responsible for energy production within the heart. Only recently have researches been able to show the interconnectedness between energy production to allow the heart to pump blood and how it can lead to heart disease. Heart disease, in turn, causes degeneration of mitochondria through free radicals further damaging the energy production machinery and causing the disease to get worse. This review will define the current definitions and understandings over the areas of cardiovascular disease, cardiac bioenergetics, mitochondria, electron transport, and reactive oxygen species. These will include up-to-date evidence on the current knowledge in each respective field. In whole, this review will attempt to show how each of these topics can be formed into a working theory on the contribution of mt-mRNA translation in maintaining cellular oxidative metabolism. This will culminate in the development of a research proposal entitled: **Mitochondrial mRNA translation is required for maintenance of oxidative capacity**.

1.Cardiovascular Disease

1.1.Definitions As defined by the American Heart Association, total cardiovascular disease (CVD) includes rheumatic heart disease, hypertensive diseases, ischemic heart diseases, pulmonary heart diseases, pulmonary circulation diseases, other forms of heart diseases, cerebrovascular diseases, atherosclerosis, diseases of the arteries, arterioles, capillaries, veins, lymphatics not classified elsewhere, unspecified disorders of the circulatory system and

congenital cardiovascular defects. Of those diseases included in total CVDs, coronary heart disease (CHD) is the most prevalent followed by heart failure. CHD includes acute myocardial infarction (MI), angina pectoralis (chest pain, AP) and heart failure (HF). The Center for Disease Control and Prevention (CDC) helps to outline defining characteristics of these specific conditions. HF, or congestive HF, results from the inability for the heart to provide adequate blood supply to other organs in the body. Conversely, MI results from the hearts inability to provide itself with sufficient blood supply due, usually, the blockage of coronary blood flow. An underlying cause and effect of many of these heart diseases is cardiomyopathy which is when the heart becomes enlarged and stiff decreasing the effectiveness of the heart pumping.

1.2.Statistics Because of the overlapping nature of many different forms of CVD and CHD, many of the statistics cannot be added together to derive a meaningful total.

1.2.1.Total CVD Total cardiovascular disease (CVD) includes rheumatic heart disease, hypertensive diseases, ischemic heart diseases, pulmonary heart diseases, pulmonary circulation diseases, other forms of heart diseases, cerebrovascular diseases, atherosclerosis, diseases of the arteries, arterioles, capillaries, veins, lymphatics not classified elsewhere, unspecified disorders of the circulatory system and congenital cardiovascular defects.

1.2.1.1.Morbidity and Mortality Greater than 85 million Americans (~26% of the population) currently have at least one form of CVD. CVD has been the primary underlying condition of more deaths every year for more than a century in the United States. For the most recent annual records, CVD caused 30.8% (801,000) of deaths in the United States and CVD was mentioned in 54% (1,402,000) of all death records. For adults >65 years of age, CVD was the leading cause of death regardless of sex and ethnicity. Greater than 155,000 Americans died before age 65 due to CVD. Greater than 1 in 3 deaths that occurred because of CVD happened before average life

expectancy was reached (78.8 years). On a global scale, CVD is still the leading cause of death and represents ~1/3 deaths worldwide[1]. Worldwide, the differences in mortality are seen between men and women but death rates are much greater in low to middle income countries[2].

1.2.1.2.Costs The estimated total costs of CVD in the United States is currently $317 billion (USD). Increases in CVD costs are projected through the year 2030 for the United States and will near $1,000 billion[3]. Similarly, an increase of nearly three times the current amount is projected for worldwide costs associated with CVD from $860 billion to > $2,000 billion by 2030.

1.2.2.Coronary Heart Disease Coronary heart disease includes acute MI, angina pectoralis and HF.

1.2.2.1.Morbidity and Mortality Total prevalence of CHD among men and women in the US is 6.2% of the population over 20 years aged. Because of the frequency of 'silent' MIs (that is MI where ischemic damage has occurred with no symptoms), there is some difficulty in estimating incidence of MI. Annual estimate of MIs in the United States is 550,000 new attacks and 200,000 recurrent attacks. Assuming ~21% of first and recurrent attacks are silent, Americans will suffer ~660,000 first time heart attacks and ~305,000 will suffer recurrent attacks. Men and women suffer CHD at different rates. Average age for first MI in men is 65.1 years while average age for women is 72.0 years. The overall incidence lags behind in women by about 10 years but for serious, life threatening or hospitalizing events such as MI or sudden death, women lag behind by ~20 years. CHD was the underlying cause of death in ~1 in 7 (370,000) American deaths. Specifically, MI contributed to ~1/3 of all CHD deaths (117,000). The estimated average years of life lost due to MI death is ~17 years.

1.2.2.2.Costs Average direct and indirect annual costs due to CHD in the United States is ~$210 billion. MI and CHD were 2 of the top 10 most expensive hospital discharge statements (~$22 billion combined). Average medical costs due to MI and CHD are expected to double in the next 10 years to nearly $500 billion.

1.2.3.Cardiomyopathy and heart failure Cardiomyopathy occurs when the striated muscle of the heart becomes stiff due to buildup of scar tissue or non-functional contractile tissue and causes the heart to become less functional at circulating blood to the organs of the body. Throughout the world, heart failure was most highly associated with hypertension[4].

1.2.3.1.Morbidity and mortality Cardiomyopathy is responsible for 23,000 deaths of Americans over the most recent year's estimates and is mentioned in almost 50.000 of the records of all-cause mortality cases. Hypertrophic cardiomyopathy is the most common heart defect and occurs in ~500,000 Americans while most are not aware of its prescience[5]. In the last two decades, the global death rate attributed to cardiomyopathy has increased substantially (41%) to >403,000 deaths. Estimated projections show that HF will increase by almost 50% in the next decale to over 8,000,000 people over the age of 18[6]. Over ¾ of cases of heart failure have antecedent hypertension. After age 40, men and women both have the same risk for developing HF at 1 in 5 adults. One in nine deaths mentions HF on the death certificate[7]. Unbeffiting disease of the Western society, HF is common in sub-Saharan Africa at 44% of patients with CVD but occurs at a younger age[8]. It is worth noting that heart failure with conserved ejection fraction (EF) is associated with a greater survival rate over fifteen years[9].

1.2.3.2.Costs In the year 2012, HF attributed to an estimated cost of ~$31 billion[6]. Projections predict that heart failure will exceed $70 billion in 2012. This results in an estimated cost $250 for every American adult.

1.3.Health Behaviors and other risk factors Risk of one developing total CVD is tightly connected to how a patient is characterized based on the AHA's newly created list of 7 metrics. These include both lifestyle health behaviors (diet quality, physical activity levels, smoking, body composition) and health measurements/biomarkers (Total cholesterols, blood pressure, blood glucose). Each of these can be treated as if there exists a spectrum of cardiovascular health of ideal, intermediate, or poor health. Additionally, many of these 7 metrics can independently contribute to disease states that are additional risk factors for CVD (i.e. diabetes mellitus, metabolic syndrome).

1.3.1.Smoking/Tobacco Use According to reports by the Surgeon General, smoking is a major risk factor for total CVD[10]. Smoking as a risk behavior is considered as an ideal behavior if one has never smoked or has quit smoking >12months ago. Additionally, risks of CHD, acute MI and stroke is increased for those who work or live in an environment with secondhand smoke. There is not enough evidence yet to determine a risk of electronic nicotine delivery systems such as e-cigarettes on CVD risk[11].

1.3.2.Physical Inactivity Getting too little physical activity is a major risk factor for CVD[12]. One in 3 children and less than half of adults meet the requisite physical activity criteria. Although distinct from physical activity, measures of cardiorespiratory health is a stronger predictor of CVD risk[13]. In 2014, just 21.4% of adults met federal guidelines for adequate physical activity. Association of physical activity and decreasing metabolic risks is maintained regardless of weight loss from the physical activity[14].

1.3.3.Nutrition Multiple factors contribute to the contribution of dietary intake to CVD risk. Specifically, a DASH-type diet (dietary approach to stop hypertension) is recommended. These guidelines include increasing whole grains (>3 servings/day), increasing fruit (>2 servings/day),

increasing non-starchy vegetables (>2.5 servings/day), increasing fish and shellfish (>2 servings/week), increasing nut and seeds (>4 servings/week), decreasing red meat, decreasing sugar-sweetened beverages, decreasing sweets and bakery desserts, decreasing saturated fats, increasing unsaturated fat and omega 3-fatty acids, increasing fiber, decreasing sodium, and increasing potassium intake. Improving healthy eating among the population is the most cost-effective approach to decreasing risk of CVD compared to smoking cessation, statin therapy, and lifestyle advice[15].

1.3.4.Body Composition Maintaining a BMI $\leq 25 kg/m^2$ is one of the 7 components of maintaining ideal cardiovascular health. Being overweight ($25 kg/m^2 \geq$ BMI $\geq 29.9 kg/m^2$) or obese (BMI $\geq 30 kg/m^2$) is considered a major risk factor for total CVD including CHD, AF, CHF, stroke, and venous embolism. Based on recent NHANES data, 69% of American adults are overweight or obese. Obese individuals pay ~$1,500 more for health care costs than normal-weight individuals including 50% more inpatient costs, ~25% more outpatient costs, and ~80% more on prescription drugs[16]. Strong evidence exists for bariatric surgery among patients with a BMI $\geq 40 kg/m^2$ though few clinical trials exist. Bariatric surgery can cause substantial weight loss, remission of DM, hypertension and dyslipidemia and reduce total medical expenses by ~$10,000 within 5 years[17].

1.3.5.Family History First degree relatives share their genetic variation much more than a randomly selected individual. Likewise, those with similar racial/ethnic backgrounds tend to demonstrate similar genetic variations within the specific population. This contributes to complex genetic factors involved in CVD risk that might be ascertained via family history of CVD. Risk of premature heart attack, acute MI, AP, angioplasty, bypass surgery, CHD, aortic fibrosis, stroke, PAD, and venous embolism are all much higher in those who have a first degree

relative with history of the respective CVD. Recent consortia have produced genetic studies of CAD associated with specific genetic loci. While low odds ratios are seen, individuals may hold up to two alleles of a single gene and multiple genes involved in CVD risk[18].

1.3.6.Blood Cholesterols High cholesterol has been considered a major risk for total CVD with ideal levels for cardiovascular health being < 200mg/dL total cholesterol for adults. Less that 50% of adults currently meet this ideal criterion.

1.3.7.Blood Pressure High blood pressure is a major risk factor for CVD and stroke. The ideal blood pressure considered ideal for cardiovascular health is <120mmHg SBP and <80mmHg DBP. ~40% of American adults currently meet this standard. Over 400,000 deaths per year mention HBP in deaths. When compared with out causes of CVD deaths such as dietary, lifestyle and metabolic risk, HBP is the leading cause of death among women and the second-leading cause among men (behind smoking[19].

1.3.8.Diabetes Mellitus Untreated fasting blood glucose level of <100mg/dL is a component of ideal cardiovascular health. DM or uncontrolled hyperglucosemia is present in >50% of adults and is a major risk factor for CVD including CHD, stroke, PAD, HF, and AF.

1.3.9.Metabolic Syndrome Combinations of cardiometabolic risks can be categorized as metabolic syndrome and can be beneficial in clinical and patient communications involving the nature and etiology of the diseases. Fasting glucose, dyslipidemia, adiposity, and blood pressure above ideal levels, when taken into combination, characterize metabolic syndrome.

1.3.10.Chronic Kidney Disease End-stage renal disease (patients undergoing dialysis or receiving kidney transplantation) increases risk for CVD. While less common, renal disease is associated with DM and HBP due to altered filtration through the kidney tubules.

1.4.Functional Assessment of Heart Disease To understand the underlying etiology of heart diseases, it is valuable to understand how a patient's heart is functioning. Electrical assessment, imaging modalities, circulating biomarkers and physical activity capacity (measured via CPET) are all ways in which one can assess various factors influencing cardiac health. The primary goal of these functional assessments is to determine the etiology of exertional fatigue and dyspnea[20]. CPET can determine where in the cardiopulmonary system is lacking while echocardiography is able to visually image the specific function of the heart that may be at fault. The most powerful tool in this regard is echocardiography due to its ability to assess systolic and diastolic function of ventricles as well as other issues[21–23].

1.4.1.Echocardiography While still undergoing refinement in the 1970s and 1980s, utilization of echocardiography to assess cardiac architecture has become an increasingly powerful tool for visualizing heart function. Initially, even with low quality sensors, much debate surrounded calculations that were viable, simple, and specific in detecting LVEF[21,24]. Many of these approaches were not sensitive in comparison to calculations that became available with improved piezoelectric crystals[23]. These and other advances would eventually lead to accurate evaluation of pulmonary hypertension, thrombus, valve dysfunction, ischemia, and viability in heart failure[25–27]. Multiple clear markers of heart failure are evident through echocardiograph assessment. Enlargement of the heart is highly associated with mortality and an important predictor of clinical outcomes in heart failure[28–32]. A stronger determinant of cardiac health, rather than chamber dimensions, is LVEF[33].

1.4.2.Cardiopulmonary exercise testing Cardiopulmonary exercise testing is quickly becoming a clinically relevant functional assessment predictive of HF prognosis[34]. Recently, the AHA has even suggested the cardiorespiratory fitness should be assessed clinically as a vital sign[35,36]. This

non-invasive testing process can determine if insufficiency in exercise capacity is due to myocardial/circulatory or gas exchange abnormalities[20,37]. Other testing methods have been used as well to predict functional capacity and mortality of those with heart disease including overall cardiorespiratory fitness[38], heart rate recovery after activity[39], a non-exercise testing method[40], assessment of 6-minute walk test[41], among other functional measurements[42].

1.5.Molecular alterations during heart disease A multitude of alterations are seen at the cellular and molecular level of cardiac cells including alteration in contractile tissues and a changing metabolic profile of the existing tissue. The common representation of the myocardium is a machine that is able to convert potential chemical energy into kinetic mechanical energy in the form of a hydraulic pump[43]. In the case of ischemic heart damage, alterations in cellular and metabolic components is due to apoptosis stimulated by hypoxia and a lack of energy substrate[44,45]. However, critics suggest this creates a circular argument in that a reduction in contractile components might propagate metabolic insufficiencies and that the true cause may be caused by transient intramyocardial bioenergetic stressors[43]. To demonstrate how cellular bioenergetics plays a crucial part in heart disease (similar to hemodynamic alterations), a review of the bioenergetic processes involved in cardiac disease and the molecular and cellular processes follows.

2.Cardiac Energy Metabolism The heart contributes less than 0.5% to total body weight but nearly 10% to total energy consumption through ATP[46] yet the amount of ATP stored in the myocardium is only sufficient to support a few heart beats[47]. Around 90% of the ATP in cardiac muscle is utilized in the contraction-relaxation cycle – both active processes. Both the release of actin from myosin during contraction/systole[48] and cytoplasmic sequestration of calcium during relaxation/diastole requires significant amounts of ATP[49]. Between 60 and 70% of ATP

hydrolysis is used in contraction which the remaining 30-40% is used for sarcoplasmic Ca^{2+} uptake[50,51]. maintain such a high metabolic turnover rate, cardiac cells contain the greatest content of mitochondria compared to any other tissue in the body at nearly 30% of cell volume[52]. *In vivo* working hearts are able to produce ATP at 80-90% of the mitochondrial OXPHOS capacity[53]. To The cardiomyocyte has also developed a sensitive array of cytoplasmic sensors (Ca^{2+}, ADP, P_i) that act as a control network over mitochondrial energy conversion to maintain myofibrillar potential energy homeostasis[54]. Mitochondria present as an integral part of cardiac energy metabolism allowing for : 1) utilization of carbon substrates, 2) the generation of high energy ATP through the electron transport system, and 3) the translocation of the ATP to the myofibrils to be used for contractile work[45].

2.1.Carbon substrate utilization Mitochondrial oxidative phosphorylation is fueled by electrons generated when carbon energy substrates are oxidized to form reduced NADH and $FADH_2$ primarily in fatty acid β-oxidation, the TCA cycle, and in small part from pyruvate dehydrogenase and glycolysis reactions[55]. Carbon fuel sources for ATP generation are stoichiometrically linked to the contractile power produced by the myocardium. In a healthy, oxygenated heart, the primary carbon substrate of the TCA cycle, Acetyl-CoA, is generated predominantly by fatty acid β-oxidation over carbohydrates from glycolysis/lactate metabolism in a range from 60%/40% to 90%/10% (fatty acid/pyruvate)[56–58].

2.1.1.Glycolytic substrates The myocardium is a net consumer of lactate even under maximal workload except for cases of ischemia[59] and insulin dependent diabetics[60]. The glycolytic substrates of the heart are circulating glucose or intracellular glycogen stores. Extracellular glucose is endocytosed in a GLUT-4 (and partly GLUT-1) dependent manner indicated susceptibility to contraction, AMPK, and insulin stimulation[61]. Internal glycogen stores can be

converted to G6P for glycolysis though cardiac glycogen stores are relatively small and turnover rapidly[62]. The end of the glycolytic process produces pyruvate which has three possible fates: becoming lactate, acetyl-CoA, or anaplerosis. Glycolytic reactions and pyruvate utilization is inversely related to plasma FFA levels as suggested by the Randle cycle or glucose-fatty acid cycle[63,64].

2.1.2.B-oxidation substrate While glycolysis substrates are either stored within the cell (glycogen) or absorbed in response to insulin, contraction, or inadequate energy (blood glucose), fatty acids are not stored and must be taken up continually. The uptake of fatty acids is dependent on the concentration of plasma nonesterified free fatty acids which is highly variable and sensitive to both acute and chronic metabolic stimuli[65–67]. Fatty acids are extremely hydrophobic and as such require close association with carrier or transport proteins to move freely (i.e. bound to coenzyme A or covalently as triglycerides). The uptake of fatty acid by the cell also requires specialized transport proteins (fatty acyl translocases, fatty acyl binding proteins) or passive diffusion from an albumin bound form in the plasma to a cellular carrier[68]. Once inside the cell, fatty acids are generally esterified into a fatty acyl-CoA which cannot be directly transported through the mitochondrial membranes to the matrix where β-oxidation can occur[69]. A series of acyltranslocases allow the passage of the fatty acyl-CoA from the cytoplasm to the matrix -- primarily controlled through carnitine palmitoyltransferase-I. Carnitine palmitoyltransferase-I is the key regulator of fatty acid import into the mitochondrion and is negatively regulated by malonyl-CoA which is generated by acetyl-CoA carboxylase[66]. Once inside the mitochondrial matrix, the fatty acyl is oxidized through β-oxidation which cleaves off consecutive two-carbon acetyl-CoA molecules producing reduced NADH and $FADH_2$[66,67].

2.1.3.Ketone bodies It should be noted that ketone bodies such as β-hydroxybuterate and acetoacetate can be utilized for energy by the myocardium. This only occurs when plasma concentration reaches levels only seen during uncontrolled diabetes or starvation and is a very minor substrate[70,71].

2.2.ATP generation through the electron transport system Electron transport will be addressed in detail in section 4 but, briefly, carbon metabolites are utilized in glycolysis, the TCA cycle, and β-oxidation to generate reduced electron carriers NADH and $FADH_2$. These electron donors are used in a series of redox reactions along each of four mitochondrial inner membrane complexes to generate an electrochemical gradient between the mitochondrial membranes. This proton motive force is utilized by F_0/F_1 ATP synthase to generate high energy ATP from ADP and P_i[72].

2.3.Translocation of ATP to myofibrils for contraction ATP molecules are generated by the ATP synthase within the mitochondrial matrix but must be transferred to the myofibrils where the majority of energy is used within the myocardium[50,51]. ATP can be easily transported outside of the mitochondrion by Adenine Nucleotide Translocase where the high energy phosphate is temporarily stored with a creatine molecule by the creatine kinase reaction. This allows high energy phosphates to be generated within the mitochondrion but then transported and utilized at the sarcoplasmic reticulum and myofibrils for the processes of contraction and relaxation. Recent studies have shown how the speed of this movement is far too rapid for simple diffusion through the cell and instead use the mitochondria as a reticulum. This allows the generation of the proton motive gradient in one location (presumably near the source of carbon substrates) and use the energy to form ATP at a distant part of the mitochondrion (near the myofibrils/sacroplamic reticulum)[55,73]. Fragmentation of the mitochondria may lead to detriments in the distance the

mitochondrial reticulum can transfer energy (see section 3.1.2). This mitochondrial reticulum is a highly regulated and dynamic concept that is heavily involved in cardiac health and disease.

3.The Mitochondrion Cardiac mitochondria are organized in such a way as to allow energy production near the cellular locale in which the energy will be used -- near the SR (subsarcolemmal), nucleus (perinuclear), or contractile apparatus (intramyofibrillar)[74]. Mitochondria are extremely dynamic organelles which were investigated as soon as the early 1900's because they're constantly being combined, separated, expanded, or destroyed[75]. Each of these processes are highly regulated and contribute to the structural and functional integrity of the organelle for optimal energy production[76]. This section will briefly detail each of these processes with particular interest in how they are affected in heart disease. It will then focus on mtDNA and the processes that allow mtDNA to facilitate proper energy production in the heart.

3.1.Mitochondria in brief The mitochondria exist as a discreet organelle with symbiotic origins that now acts as the primary source of oxidative energy production in eukaryotic cells[77]. The mitochondria contain an outer membrane sectioning the organelle from other cellular components and an inner membrane that form cristae to increase surface area and form a discreet internal matrix. Within the mitochondrial matrix are the necessary enzymes for the processes of β-oxidation, the TCA cycle reactions, redox handling proteins, mtDNA and proteins to allow mtDNA transcription and translation as well as numerous other regulatory and functional processes. Integrated within the inner membrane are transporter proteins and the ETC complexes which use electron donors (NADH and $FADH_2$) in a series of redox reactions to move protons from the mitochondrial matrix to the intermembrane space. This generates an electrochemical gradient or a proton motive force that is utilized by the F_0/F_1 ATP synthase to create energy.

3.1.1.Fusion of mitochondria Within a cell, multiple mitochondria may combine by joining their inner and outer mitochondrial membranes (IMM and OMM, respectively). This allows more efficient and homogenous sharing of components of the matrix and combining the electrochemical gradient within the intermembrane space[78]. Mitochondrial fusion proteins can contribute to larger, more stable and efficient mitochondria and thus energy production – vital to maintain oxidative metabolic potential during pathologies associated with heart disease. The combination on the membranes is primarily controlled by the protein mitofusin-1, mitofusin-2 (both for OMM fusion), and optic-atrophy-1 (for IMM fusion)[79,80]. The expression levels of these proteins is sufficient to control the process of mitochondrial fusion but can be further modified through post-translational modifications including phosphorylation and ubiquitylation/degradation[81]. Mitochondrial fusion proteins seem to be particularly susceptible to ROS during reperfusion injury, are downregulated in diabetes and can dictate the preference for metabolic substrate[82–84].

3.1.2.Fission of mitochondria Mitochondrial fission is required to maintain a balance with mitochondrial fusion to maintain shape, size, number, and homogeneity of mitochondria populations[85]. The primary controller of mitochondrial fission is dynamin-related protein 1 (DRP-1). DRP-1 is a GTPase (similar to each of the fusion proteins) that undergoes the catalytic activity to break apart the IMM and OMM of separating mitochondria. A key difference lies in that DRP-1 does not contain a mitochondrial membrane anchoring sequence and must be recruited from the cytosol to the OMM and bound to one of a few assembly proteins. These OMM proteins are key to recruitment of DRP-1 and aid in the process of mitochondrial fission: mitochondrial fission protein, mitochondrial fission factor, and mitochondrial dynamics proteins 49 and 51[86]. Calcium induces the translocation of DRP-1 to the OMM and is seen prominently in

reperfusion injury. Mitochondrial fission is strongly linked to mitochondrial deterioration during insulin resistance in skeletal muscle but has not been shown in cardiac muscle[87]. Finally, chronic alterations in mitochondrial fission can affect a population of small, isolated mitochondria that are a target for the process of selective autophagy.

3.1.3.Autophagy of mitochondria It was recently revealed, in 2008, that selective fission and fusion of mitochondria creates two segregated populations. One group presents with disrupted membrane potentials and are subsequently broken down through the process of autophagy[88]. After further research in yeast and mammals, the process of mitochondrial specific autophagy or mitophagy was characterized. Beclin1 is a protein found on the initiated phagophore and during elongation, LC3 becomes incorporated into the phagophore. LC3 is a key protein on the developing phagophore that can bind specific OMM proteins and lead to complete destruction of the separated portion of the organelle through marcoautophagy. LC3 interacting molecules that have been identified including BNIP3/NIX, FUNDC, cardiolipin (when present on OMM), and Parkin recruited p62[89,90]. By selectively destroying damaged or mutated mitochondria, a cell can protect itself from accumulation of ROS and somatic mtDNA mutations that can be deleterious to the cell[91]. As yet, no study has specifically determined the functions of mitochondrial specific autophagy in cardiomyocytes though it has been seen that in Atg5 KO mice (loss-of-function for general autophagy), cardiac disease is present with oxidative maladies[92]. During cardiac stress (ischemia/reperfusion), autophagy seems to play an important role and may contribute to cardiac protection[93,94]. These studies, while opening the door for further research, indicate that mitophagy plays an invaluable part in the protection against heart disease by removing damaged and ineffective mitochondria from the cell.

3.1.4.Biogenesis of mitochondria As we've seen, mitochondria make up a large proportion of the mass of cardiac cells to allow constant energy production. During embryonic development, glucose is a preferred fuel source but during stem cell maturation, mitochondria become more abundant thus the cell can rely more on fatty acids for energy through adulthood[95]. This process of generating new mitochondria during early development is termed mitochondrial biogenesis. In the myocardium, a surge of mitochondrial biogenesis at birth allows the development of a remarkably dense array of mitochondria packed tightly between sarcomeres[96–98]. After this developmental stage and into adulthood, mitochondrial biogenesis is far less important compared to maintenance of fatty acid oxidation capabilities of existing mitochondria[99]. As mentioned, occasionally, mutations or disruptions in membrane potential warrant fission of small portions of a mitochondrion and selective destruction by autophagy. As this occurs, cardiac cells would progressively lose oxidative capacity over the lifespan if this same process of mitochondrial biogenesis did not replace functional organelles. Three groups of transcription factors appear responsible for the replication of mitochondria from existing mitochondria (rather than de novo): PPARs, ERRs, and NRFs. PPARs appear to aid in the regulation of genes involved in mitochondrial (and peroxisomal) fatty acid oxidation[100]. ERRs are a family of nuclear receptors that are involved in most mitochondrial metabolic pathways including TCA cycle components, β-oxidation, and oxidative phosphorylation[101]. NRFs are primarily involved in the regulation of genes controlling the OXPHOS components[102]. PGC-1α is a transcriptional coactivator that can coordinately activate each of these transcription factors and stimulate mitochondrial biogenesis in the cardiac muscle cell[103,104]. During pathological cardiac remodeling, mitophagy seems to play a key role in controlling ischemic damage but less is known about how mitochondrial biogenesis restores the damage organelles[93]. Speculation puts mitochondrial biogenesis in an

important position during exercise stressors, pregnancy responses, preconditioning and carioprotective processes in the heart muscle cell[97].

3.2.Mitochondrial Genetics Mitochondria are a defining characteristic of eukaryotic cells and result from an ancient α-proteobacterial endosymbiotic interaction[105]. This helps explain the presence of a circular genome within the mitochondria (mtDNA) that is highly variable between species[106]. The mtDNA has shortened dramatically and independently throughout genetic lineages with most of the still necessary genes having been transferred from the mtDNA to the nDNA. Evidence supports the evolutionary advantage of transferring genes from mtDNA to nDNA to mitigate mutations by allowing sexual recombination and lessened proximity to oxidative stressors[107]. Still, not all genes encoded by mtDNA can be successfully expressed from the nucleus[108]. This presents key questions as to the importance of the genes expressed by the mtDNA, and how to control of replication, transcription and translation of the mtDNA.

3.2.1.The mitochondrial genome Mitochondrial DNA has shown extensive alterations phylogenetically in the eukaryotic domain but remain relatively unchanged within kingdom *Metazoa*[106,109]. A high degree of homology exists between mammals, especially mouse and man[110,111]. Human mtDNA is ~16.6kb, double stranded (a heavy and a light strand based on base composition), and encodes 37 genes comprised of two rRNAs, 22 tRNAs and 11 mRNAs. Two of the mRNAs are bicistronic and therefore, each becomes 2 proteins making the total number of mtDNA-encoded proteins thirteen[112]. Maintenance and expression of the mtDNA is vitally important in mammals. Maintaining the discreet genome and associated machinery for expression seems to evolutionarily outweigh the benefit of transferring the sequences for 13 mtDNA encoded proteins to the nDNA because over one quarter of the mitochondrial proteome is involved in DNA maintenance, replication and expression[113,114].

3.2.2.mtDNA Replication mtDNA replication is distinct from that of nDNA replication and as such has specialized replication machinery. mtDNA replication machinery has been related to the replication machinery of early bacteriophages[115]. A specialized DNA polymerase-γ maintain DNA binding, exonuclease, and lyase activity, TWINKLE aids in helicase unwinding of the mtDNA, and RNA polymerase contributes the primers for the polymerase process[116–120]. The light strand promoter region is ~200bp 5' of the heavy strand origin of replication allowing for a 3' tail from RNA polymerase to act as the primer initiating heavy strand DNA replication. The primer is later cleaved by nucleases while replication continues[121]. MtDNA copy number is under control of multiple replication abortive processes but can be stimulated by overexpression of TWINKLE in mouse muscle and heart[122]. mtDNA is compacted into nucleoid structures similar to that of bacterial genomes to allow a more densely packed organization. The primary molecule forming the nucleoid structures is TFAM capable of binding the mtDNA every 16-17bps[123]. The control of mtDNA compaction by TFAM is relevant to both levels of mtDNA replication and transcription[124].

3.2.3.mtDNA repair mechanisms mtDNA is in relatively close proximity to redox centers and mutation causing free radicals (as discussed in section 5). However, little evidence suggest that a faithful or dedicated repair process for mtDNA is present. However, some evidence does suggest a limited ability for mtDNA mutations to be corrected. Research has shown that base excision repair is partially active within the mitochondria and helps mitigate oxidative DNA damage. Mismatch repair mechanisms are lost from nDNA to the mtDNA but Polγ (the only polymerase located within the mitochondria) has a specific subunit for proofreading thus minimizing the need for mismatch repair. Base dimerization and crosslinking mutations cannot be repaired effectively. Some ligase activity does occur because blunt ends and linear fragments can be

rejoined. Certain proteins have been identified that function in these processes with nDNA and are localized to mtDNA. As yet, it does not appear that a robust mtDNA repair system is active within the mitochondria. Because no recombination, if a somatic mutation occurs it will: 1) be silent, 2) prevent transcription or replication and be destroyed, 3) create mutated genes/proteins and propagate to daughter mitochondria. These mutation, especially in a mothers' egg, can create diseases with a wide array of clinical symptoms. Other theories suggest that a slow rate of mutation over lifespan can reduce oxidative potential of the mitochondria and cause aging and death[125–131].

3.2.4.mtDNA Transcription MtDNA contains two anti-parrellel strands with the outer strand being slightly heavier due to a higher proportion of guanine base pairs and thus is referred to as the heavy strand (while the complimentary is the light strand)[132]. The mtDNA is tightly packed with only one non-coding region used to regulate transcription of each strand with the LSP and HSP. Beginning transcription in this non-coding region produces mRNA transcripts that are near the length of the entire genome with termination sequences stopping transcription before completing circumscription. These polycistronic transcripts produce multiple mature mRNAs that are processed co-transcriptionally and seem to use intermittent tRNAs to 'punctuate' or flank the rRNAs and mRNA coding sequences[133]. The molecular machinery used for mtDNA transcription is similar to that of replication and involved a mtDNA-directed RNA polymerase ($_{mt}$Pol), a subunit of DNA polymerase-γ, and TWINKLE helicase activity[134]. $_{mt}$Pol was identified based on sequence homology to yeast and the x-ray structure identifies an N-terminal promoter-binding region and a C-terminal catalytic domain[135–137]. $_{mt}$Pol is able to bind the promoter region but is not able to begin transcription without additional transcription factors. Mitochondrial transcription factor B2 (TFB2M) is required to aid in the initiation of transcription

but is dissociated from the Polymerase complex after leaving the promoter region and beginning elongation[138–140]. Transcription factor A (TFAM) additionally aids in the transcription initiation complex by its role in DNA binding and compaction into a U-turn shape without sequence specificity. These shapes aid in denaturing a small portion of the mtDNA which can create an area for transcription initiation on the promoter region[141–143]. Transcription initiation models suggest that TFAM first binds ~70-75bp upstream of the start sequence creating a bend in the DNA and associating with $_{mt}$Pol to recruit it to the promoter site. As it binds ~50bp upstream of the TSS, $_{mt}$Pol recruits TFB2M to surround the TSS and help generate the first phosphodiester bonds, the rate limiting step in transcription[144]. Transcription elongation is aided by mitochondrial transcription elongation factor that aids in polymerase activity especially where secondary RNA structures or oxidative damage on DNA basepairs (8-Oxo-2'-deoxyguanosine)[145,146]. Mitochondrial transcription termination was thought to be controlled by a family of termination factors (MTERF 1-4) however only MTERF1 seems to play a role in transcription termination. The H-strand seems to have a short and long transcript size that transcribes the entire H-strand or just the first 4 genes with MTERF1 controlling the early termination for yet unknown reasons[147–149]. The role of MTERF2,3 and 4 have been identified but even with sequence similarity and sequence specific DNA binding motifs, evidence suggests they have now taken on roles in replication initiation and ribosomal assembly[150–152].

3.2.5.mt-mRNA Translation Of the >250 proteins localized to the mitochondria to aid in DNA processing, transcription and replication, they are all required because of the necessary outcome of mitochondrial translation and the few (13 in mammals) necessary proteins[153,154]. This requires maintenance of the requisite translation machinery including mRNAs, tRNAs, and ribosomes but huge disparities exist between cytosolic, bacterial, and mitochondrial translation. Almost all of

the requisite proteins for mitochondrial translation, across all species, are encoded by nDNA and must be imported into the mitochondria[155].

3.2.5.1.tRNAs While mitochondria originated form a common ancestor, many of the components of translation are substantially different between animals and bacteria. A primary example is the difference in the mammalian mitochondrial genetic code from that of the universal genetic code[156]. The mitochondrial code in mammals contain more stop codons and an extra Trp codon compared to the universal norm and contain fewer tRNAs than would be predicted with the traditional wobble rule. Among Metazoans, the majority of mt-tRNAs contain a traditional cloverleaf shape whereas some other eukaryotes contain simplified codon/anti-codon fragments only[157,158]. This is most apparent when the structures for the mitochondrial ribosomes was revealed and showed striking differences among species.

3.2.5.2.rRNAs and mitochondrial ribosomes The structure of mitochondrial ribosomes, or mitoribosomes, has changed dramatically across evolution but maintains similarity among higher organisms[159–161]. The mammalian mitoribosome can sediment as a 55S monosome consisting of a 28S SSU and a 39S LSU which contain a 12S and 16S mt-rRNA, respectively[162]. Bacteria maintain a 5S rRNA which is involved in coordination of the LSU and SSU during translation[163]. Modern mitochondria do not contain this small rRNA and instead, have evolved the interacting regions of the ribosomal subunits to compensate[164]. Despite the differences seen among mitoribosomal architecture, the catalytic active site remains constant between bacteria and mitochondria explaining side effects of many antibiotic treatments[165]. An additional responsibility of the mitoribosome is insertion of the nascent protein into the IMM. Each of the proteins encoded by mt-DNA are IMM proteins in the OXPHOS and are highly hydrophobic which may contribute to reasons why they cannot be encoded by nDNA[108]. The exit tunnel of

mammalian mitoribosomes has developed a similarly hydrophobic protein lining to mimic the protein folding necessary for membrane insertion[159]. Additionally, a ribosomal interatcting protein is anchored within the IMM and binds near the exit tunnel of the mitoribosome. Lastly, specific proteins such as Oxa1 interact with the nascent chain and acts as a protein insertase for the IMM though it Is not absolutely required[166,167].

3.2.5.3.Translation initiation Protein synthesis is an energetically expensive process and as such, translation initiation is generally a committal step. To begin translation requires specific interactions of all proteins and RNA molecules involved. In bacterial and eukaryotic translation, the process has been reconstructed *in vitro* using just this minimal necessary components but this has not been fully achieved using mitochondrial translation machinery alone[168]. Still, these experiments have shed much light on how the translation initiation process takes place. The working theory is that a preinitiation complex made up of the combined 55S mitoribosome is bound by mtIF3 which dissociates the 28S SSU and 39S LSU revealing a binding site for mtIF2 on the 28S SSU. mtIF2 is able to recruit the fMet-tRNA bound to the start codon on the mRNA to the P site within the 28S SSU. mtIF2 then hydrolyzes GTP to GDP allowing the 39S LSU to join by removing the two initiation factors. This generates the initiation complex consisting of the 55S mitoribosome, and mRNA bound by the appropriate aminoacylated tRNA[153].

3.2.5.3.1.mtIF2 Comparisons are generally made between mammalian mtIF2 and IF2 of bacteria because it is no more dissimilar than that of yeast mtIF2. Mammalian mitochondria only contain one Met-tRNA used for both initiation and elongation. A Met-tRNA transformylase has been identified that generates a subset of fMet-tRNAs to be used for initiation while the non-formylated Met-tRNA bind to mtEF-Tu for elongation[169]. mtIF2 will bind fMet-tRNA at a subdomain labeled VIC2. Domain III of mtIF2 is responsible for the binding to the 28S SSU

though this association constant is highly dependent on the presence of GDP. Because of this reliance on GDP, Domain IV removal eliminates 28S binding capability as the GDP binding domain[170]. Bacterial and eukaryotic cytoplasmic translation initiation utilize 3 'universal' translation initiation factors (1,2, and 3) however this is not the case for the mitochondria. No equivalent of IF1 has been identified in mitochondria. There is a 37 amino acid insertion after Domain V of the mtIF2 protein that shows similar activity of the IF1 protein in prokaryotic systems. In an *E. Coli* system, mutants lacking IF1 and IF2 were lethal but could be rescued by replacing mtIF2 showing that within the mitochondria, a single initiation factor does the work of two bacterial initiation factors[171,172]. The function of bacterial IF1 that was taken on by mtIF2 is that of binding the 30S SSU covering the A-site until the fMet-tRNA is bound and the preinitiation complex is formed[173].

3.2.5.3.2.mtIF3 mtIF3 shows only a small degree of homology with bacterial IF3 and no homolog has been identified in *Saccharomyces cerevisiae*[174]. The protein has not been isolated from mitochondria but sequence prediction indicates C-terminal α-helical regions similar to that seen in *E. Coli* and other bacteria as well as a flexible inter-domain linker region. The N-terminal portion is predicted to be unstructured in solution but develop a structure once associated to the ribosome[175–177]. This flexible linker is thought to play a role in its ability to bind the 55S mitoribosome and dissociate the large and small portions to all mRNA and fMet-tRNA binding to the P-site. mtIF3 is also able to stop translation from proceeding when an improper start codon is placed in to the ribosome, or when fMet-tRNA associates with the P-site without a bound mRNA[178]. This activity acts as a proofreading step preventing mt-mRNA translation initiation under inappropriate mRNA/tRNA combinations. Which mitoribosomal proteins are targeted and bound by mtIF3 and their location in the mitoribosomal subunits is not yet known[179].

3.2.5.4.Translation elongation Mitochondrial translation elongation is highly conserved from prokaryotes to mammals[180]. Elongation proceeds when a ternary complex consisting of mtEF-Tu, an aminoacylated-tRNA, and GTP associates with the A site and forms a complimentary codon:anticodon interaction. GTP is hydrolyzed to GDP by mtEF-Tu to generate a new peptide bond elongating the nascent chain. mtEFG-1 subsequently binds to the mitoribosomal complex at the A site, hydrolyzes GTP, and translocates the nascent peptide to the P site opening the A site for the next mRNA/mtEF-Tu/GTP ternary complex. Simultaneous to mtEF-G1 GTPase activity, mtEF-Tu interacts with mtEF-Ts to aid in the removal of GDP and replace the GTP on mtEF-Tu to allow subsequent recycling[181–188].

3.2.5.4.1.mtEF-Tu mtEF-Tu was originally identified because of high expression in tumor cells in 1995 and was characterized in *Bos taurus*[189]. The gene has only a short mitochondrial targeting sequence and results in a 45kDa protein that has high homolog with the bacterial counterpart. This is consistent with the mostly conserved process of translation elongation from bacteria to mitochondria[190]. The crystal structure of mtEF-Tu reveals 3 domains and a strong interaction with mtEF-Ts. Domain I binds GTP/GDP, domain II binds aa-tRNA and the mitoribosomal SSU, and domain III further interacts with the 5' stem of the aa-tRNA[191–193]. These features allow bovine mtEF-Tu to function well in *E. Coli* but the conformational changes associated with tRNA codon:anticodon binding and Domain I are not strong enough to catalyze GTP hydrolysis[194].

3.2.5.4.2.mtEF-Ts The primary function of mtEF-Ts is to aid in guanine nucleotide exchange with mtEF-Tu. The crystal structure reveals extensive binding between mtEF-TS and mtEF-Tu at three particular domain interactions[191]. Further experiments nucleotide exchange in mtEF-Tu through the aid of mtEF-Ts requires multiple important steps including removal of bound Mg^{2+},

stabilization of GTP at the β-phosphate, and conformational changes that affect the hydrogen bonding with the mitoribosome and aa-tRNA[195–198].

3.2.5.4.3.mtEF-G1 mtEF-G1 is the main protein controlling the translocation step of mitochondrial translation elongation and involves GTP hydrolysis activity[199,200]. The GTPase activity requires specific activity from portions of the mitoribosomal LSU[200] but very few other enzymatic experiments have been performed limiting out knowledge on the functional characteristics of mtEF-G1[201].

3.2.5.5.Translation termination In mitochondria, UAA and UAG serve as stop codons while UGA is replaced. When the stop codon enters the A site of the 55S ribosomal complex, it is recognized by the GTP bound mtRF1a activating the GTP hydrolysis to allow the release of the now completed polypeptide chain. Subsequently, mtRRF1 and mtRRF2 (mitoribosome recycling factors) dissociate the two mitoribosomal subunits to allow for ribosomal recycling[202].

3.2.5.6.Translation Activators Translational activators were first reported in 1989 and 1990 from yeast models where research identified a nDNA-encoded protein that localized to the mitochondria and activated translation[203–206]. Translational activators are able to enhance mt-mRNA translation by binding (with sequence specificity) to mt-mRNAs, the mitoribosome, and tethering to the IMM where the nascent peptide is to be inserted[207]. These translational activators have implication on feedback loops and in controlling the number of key subunits for OXPHOS components. The only known human translational activator is responsible for COX1 translation and assembly[208,209]. An additional mechanism implicated for activation of mitochondrial translation is through microRNAs interacting with AGO2[210]. These results are controversial and have yet to be confirmed because other transcriptomic approaches have not identified microRNA enrichment in isolated human mitochondria[211].

3.3.Mitochondrial DNA-encoded proteins The 13 proteins that are generated as a result of the mtDNA expression system are necessary to generate the OXPHOS system[212]. These mt-DNA encoded proteins are assembled with proteins from the nDNA into the IMM and allow the OXPHOS system to function. These proteins are incorporated into subunits I, III, IV, and V[213]. Further detail on the function of these proteins within the OXPHOS complexes and the ETS as a whole follows.

4.Electron Transport and Oxidative Phosphorylation The chemiosmotic theory of ATP synthesis in cholorplasts and mitochondria was originally proposed by Peter Mitchell in 1961[214]. This model explained that a proton gradient formed across a membrane space and that the electrochemical potential energy that formed across this membrane could be used to drive phosphorylation of ADP to generate ATP. This provided for a mitochondrial model accounting for a series of electron transfer reactions which electrons from O_2 to produce H_2O with the subsequent energy yield moving protons across the IMM against the concentration gradient. This provided an ATP-less mechanism to generate the gradient for the ATP synthase pump to make ATP[215,216]. This findings were confirmed by the development of a method to reconstitute the proteins into a biological membrane and measure the rates of proton pumping using pH indicators[217]. The OXPHOS system consists of: 3 proton pumping complexes (I, III, and IV), succinate dehydrogenase, two electron carriers (coenzyme Q/ ubiquinone and Cytochrome C), and finally the ATP synthase energy generating pump.

4.1.NADH:ubiquinone oxidoreductase Complex I of the ETC completes the overall reaction of transferring electrons from NADH to ubiquinone by a series of redox reactions to pump protons across the IMM. Complex I is made up of 45 distinct protein subunits though 14 of these are highly conserved and sufficient for energy transduction. Of the 14 core proteins, seven are

hydrophilic and extend into the matrix of the mitochondria and are encoded by nDNA while the other seven create a hydrophobic intermembrane arm extends through the IMM. Each of the seven core subunits within the membrane arm is encoded by mtDNA and translationally inserted into the membrane. The seven mtDNA-encoded proteins are: ND1, ND2, ND3, ND4, ND5, ND6, and ND4L. These molecules contain between three and 16 transmembrane helices and thus are extremely hydrophobic, possibly contributing to their conservation in mtDNA. It is predicted that these ND subunits create antiporter-like half channels through the membrane and allow transfer of protons halfway connecting with a subsequent half channel to complete the translocation. These channels are thought to be filled with water molecules and act with a Grotthus–like mechanism whereby the protons are not translocated but rather exchanged between water molecules to allow more rapid movement. This would allow uncoupled leakage of protons across the membrane if no gating took place. The energy to use these channels against a gradient is generated distally from the ND channel subunits. In the hydrophilic arm within the matrix, NADH is oxidized to NAD^+ transferring the electron to Flavin mononucleotide generating $FMNH_2$. The oxidized NAD^+ is returned to the mitochondrial matrix to replenish the pool of substrates for the TCA cycle or β-oxidation. The reduced $FMNH_2$ transfers its electron to a series of seven FeS clusters that rapidly transfer the electron from the periphery of the complex towards to membrane-bound portion. At the FeS cluster nearest the membrane, ubiquinone accepts the electron and becomes the reduced ubiquinol. An α-helical subunit of the complex runs lengthwise from the electron transport centers to the membrane bound arm and is theorized to aid in the coordination of electron transfer to proton pumping. The $FMNH_2$ intermediate can react with molecular O_2 to produce O_2^- (superoxide) because of the low reduction potentials of the FeS clusters within complex I. Ubiquinone is reduced to ubiquinol (a 2-electron reaction) by

2 one-electron steps involving a semi-quinone intermediate. The predicted stoichiometry moves 2 electrons from NADH to ubiquinone (generating NAD^+ and ubiquinol) and allows the movement of ~4 protons from the matrix to the intermembrane space[218].

4.2.Succinate dehydrogenase Succinate dehydrogenase is considered an enzyme within the TCA cycle as well as complex II of the ETC complexes. The complex is made of four subunits tethered to the IMM. The subunit SDHA catalyzes the succinate to fumerate redox reaction generating the electrion donor $FADH_2$ as part of the TCA cycle. SDHB is able to accept $FADH_2$ from the SDHA reaction or from other sources such as β-oxidation. SDHB transfers two electrons from $FADH_2$ through three FeS clusters to a heme prostetic group within the SDHC/D membrane-bound portion of the complex. The heme prostetic group interacts with ubiquinone allowing two subsequent single electron reactions to generate ubiquinol similar to complex I. Complex II is far smaller than the other ETC complexes and contains zero proteins encoded by the mtDNA. Additionally, it only accepts electrons from $FADH_2$ and does not pump any protons during the electron transfer meaning it does not contribute to the electrochemical gradient across the IMM. This explains why $FADH_2$ contributes less to ATP production than NADH[219]. The reduced ubiquinol molecules from complex I and II are able to transfer electrons further down the ETC to complex III.

4.3.Cytochrome *bc*$_1$ oxidase Complex III of the ETC is also appropriately named cytochrome bc_1 (due to the reaction intermediate) or ubiquinol:cytochchrome C oxidoreductase (based on the overall redox reaction). In mammals, this complex is composed of eleven protein subunits of which three subunits are necessary for electron transport function: Cyt b, Cyt c_1, and ISP. Cyt B is the only protein of this complex that is encoded by mtDNA while the rest are encoded in nDNA. Cyt B is completely embedded within the IMM and contains two of the key redox

intermediates as b-type heme prosthetics, heme b_L and heme b_H. The other two core proteins are Cytochrome c_1 (containing a c-type heme prosthetics) and an iron sulfur protein (ISP, containing the FeS clusters). The complex functions by undergoing two rounds of the Q-cycle reactions. The first series transfers two elections from a ubiquinol molecule (product of Complex I and II) – one to a FeS cluster and another to heme b_L to transfer two protons. The electron from the FeS cluster is passed to the heme *c* prostetic on cyt c_1 and then to a membrane diffusible carrier cytochrome C. The electron on the heme b_L is transferred to heme b_H and then to an oxidized ubiquinol to create a semiquinone intermediate. A second round of this cycle will take place to further reduce the semiquinone to ubiquinol and pumping two more protons across the IMM. In total, 4 protons are pumped to the intermembrane space, 2 cytochrome c molecules are reduced, and 1 ubiquinol is oxidized to ubiquinone. The Q-cycle will continually to transfer electrons to/from ubiquinol/ubiqinone/semiquinone. This ceaseless transfer of electrons will cause some leakage of electrons to O_2 generating O_2^-. This occurs to a low extent during normal electron transport, to a greater extent during excess ETC reduction, or if the transfer of electrons to the FeS clusters is blocked by an inhibitor such as Antimycin A. Both the semiquinone intermediate and the initial transfer of electron into the complex occurs with the heme b_L acceptor in the Cyt b protein – the sole protein encoded within mtDNA[220]. The reduced cytochrome c molecule diffuses in the IMM to complex IV for further electron transport.

4.4.Cytochrome *c* oxidase Complex IV of the ETC oxidizes cytochrome c from the reactions of complex III and transfers the electrons to O_2 to form H_2O. The complex is composed of 13 subunits of which the three core subunits are encoded by mtDNA and are responsible for electron transport. The three subunits encoded by the mtDNA are the largest and most conserved showing homology to bacterial proteins. Similar to complex I and III, the mtDNA encoded

subunits make up the functional components of the complex. In complex IV, COXI (subunit I) contains $heme_a$, $heme_{a3}$, the copper center Cu_b, and the molecular O_2 that is to be reduced to H_2O. COXII (subunit II) contains the Cu_a center. The function of the final mtDNA-encoded subunit, COXIII is less understood. In a redox cycle of complex IV, cytochrome c (from reduced from complex III) transfers two electrons to Cu_a on COXII, then to $heme_a$, $heme_{a3}$, and Cu_b on COXI then finally to oxygen forming H_2O and resulting in 4 protons being pumped to the intermembrane space. The full series of reactions uses 4 reduced Cytochrome C donors, one molecule of O_2, and 8 protons to generate $2H_2O$ molecules and pumping the remaining 4 protons across the membrane. While Complexes I and III are generally accepted as the primary, physiological sources of ROS production (and potentially Complex II through new evidence), Complex IV has long been controversial as it relates to production of O_2^-. Generally, Complex IV does not produce any physiologically relevant amount of ROS but can be a contributor under hypoxic stress as the redox centers become completely reduced[221–223]. This is the final complex associated with electron transport to create the proton motive force across the IMM. To couple the electron transport to oxidative phosphorylation requires proper functioning of the F_0/F_1 ATP synthase, or Complex V.

4.5.F_0F_1 ATP Synthase ATP synthase, or sometimes ATPase is the fifth and final complex of the OXPHOS system. ATP synthase is an enormous protein complex with a complicated structure involved a membrane bound portion, a matrix, soluble portion, and a 'stator' connecting the two. The central, rotating F_1 portion bind ADP and P_i and rotates to combine the two into ATP before releasing the newly generated energetic molecule. This F_1 portion is linked to the F_0 membrane bound portion which rotates in response to protons flowing through a membrane channel, with the concentration gradient. This flow of protons along the concentration

gradient acts to rotate the c subunit ~20-30^{o} per proton. This rotation is transferred to the F_1 portion through a γ[epsilon] neck resulting in ~120^{0} of rotation in the F_1. This is sufficient to allow a conformational change of the ADP + P_i binding subunits into a tight confirmation generating ATP. After another series of protons is moved, and the F_1 portion continues to rotate, the ATP will be released allowing subsequent ADP phosphorylation. Of the numerous proteins involved in this complex molecular engine, two subunits are encoded by mtDNA in mammals. The genes *ATP6* and *ATP8* encode the ATP synthase proteins mt-ATP-a and mt-ATP-A6L. Similar to the other mtDNA-encoded proteins, these are extremely hydrophobic and integrated deep within the membrane. They also seem to play key roles in the function of the protein complex. Subunit a is a primary transmembrane component of the proton channel that allows the H^+ to travel across the IMM. Subunit A6L contributes to this as well by connecting subunit A to the F_0 subunit c which is rotating in response to proton movement[224–231]. These are the last two protein examples encoded by the mtDNA and finalize the continuing theme of the primary importance of the mitochondrial genetic machinery to maintain aerobic life.

5.Reactive Oxygen Species As described above, electrons move quickly and coordinately along the ETC to facilitate the development of the proton gradient ($\Delta\psi_m$) from ATP synthesis. At certain key transfer steps, electrons can react with unintended acceptor molecules. Molecular O_2 is able to act as a single electron acceptor generating O_2^-(superoxide). Superoxide then rapidly a dismutase and H_2O to generate H_2O_2 **(**hydrogen peroxide). The instability of the peroxide bond results in H_2O_2 having high reactivity and readily transferring an electron to any acceptor molecule so that it can be oxidized back into H_2O. Superoxide, hydrogen peroxide, among other unstable electron donors are termed reactive oxygen species (ROS) because of their propensity to oxidize and transfer a free electron radical to a reducing molecule. This section will discuss

sources of O_2^- within the mitochondria, deleterious effects of excessive ROS and mechanisms to mitigate free radicals using anti-oxidant defenses.

It should be noted that this section details the production of superoxide and its products from mitochondria as a source. Other forms of free radicals exist from sources such as cytoplasmic xanthine oxidases, catechols, transition metals (Cu^{2+}, Fe^{2-}), membrane lipooxygenases, prostaglandin synthesis, lysosomal peroxidases, peroxisome oxidases, and NADPH oxidases.

5.1.Production of superoxide Diatomic oxygen is the final electron acceptor of the ETC to be transformed into water but when O_2 accepts a single electron, it becomes superoxide by univalent reduction. This is the main ROS species of interest and is the direct result of imperfect redox exchanges along the IMM. Other species of ROS are generally formed through subsequent interactions with O_2^-. The primary sources of O_2^- have been identified as Complex I and III of the ETC and this can occur through multiple reactions. Categorically, the source of the electron donor within Complex I or III suggests the redox state and $\Delta\psi_m$ that could be expected to result in excess O_2^- production: either a NADH/NAD^+ isopotential source or Q/QH_2 (ubiquinone/ubiquinol) isopotential source[232–234].

5.1.1.NADH/NAD^+ isopotential A group of redox enzymes is capable of producing O_2^- based solely on the redox state of NADH/NAD^+. These reactions involve a flavin molecular intermediate to assist in the redox reactions which is the source of the electron singlet. This mode of ROS production might be seen in mitochondria undergoing very low ATP production but with high amounts of energy substrate such as NADH. [NADH] is generally downregulated under physiological conditions to prevent this from occurring naturally. It can be seen experimentally by inhibiting electron flow to ubiquinone as with rotenone. These [NADH]/[NAD^+] sensitive

reactions are: 1) Complex I Flavoprotein reduction, 2) pyruvate dehydrogenase, 3) branched chain keto acid dehydrogenase and 4) α-ketogluterate dehydrogenase. Predictive experiments show that complex I FMN does not relatively contribute much to the total superoxide pool compared to the other enzymes in this group. In fact, α-ketogluterate dehydrogenase is predicted to produce 8 times as much O_2^- as FMN from complex I.

5.1.1.1.Mechanisms of O_2^- generation The complex I acceptor of NADH electrons is a Flavin mononucleotide (FMN) and if FMN becomes fully reduced (such as occurs when the ubiquinol is fully reduced through increased [succinate] or rotenone), the FMN will transfer electrons to O_2^- because the $FMNH_2$ is exposed to the aqueous phase. Pyruvate dehydrogenase (PDH) is under allosteric control of NADH such that under highly reduced conditions, [NADH] is increased and activity of PDH is decreased. This allosteric regulation prevents the flavin prosthetic group from donating an electron to NAD^+. When PDH is fully reduced, this will result in the electron singlet reducing O_2 to form superoxide. Branched chain keto acid dehydrogenase catalyzes branched chain amino acids for entry into the TCA cycle. This enzyme shows a similar mechanism of superoxide generation as PDH. It is not under direct control of [NADH] but upstream molecular activators are involved in metabolic sensing. α-ketogluterate dehydrogenase uses a similar flavoprotein as PDH but is under tight allosteric control of NADH.

5.1.2.Q/QH_2 isopotential As mentioned in Section 4, Complex I and II transfer electrons to a carrier molecule ubiquinone and ubiquinol subsequently reacts at the Q-cycle within Complex III. This major electron transfer point does contain a other, less significant electron donors to the ubiquinol pool: Electron transfer flavoprotein:ubiquinone oxidoreductase (ETFQO), dihydroorotate dehydrogenase (DHODH), proline dehydrogenase (ProDH), G3PDH,

Succinate:quinone reductase (SQR) in addition to the canonical reactions of SDH (complex II) and complex III.

5.1.2.1.Mechanism of O_2^- generation For each of these redox controllers, an electron is transferred to the oxidized ubiquinone to generate a semiquinone intermediate or reduced ubiquinol. Under circumstances of excessively high $\Delta\psi_m$, concentration of reduced ubiquinol can increase and cause the Q/QH_2 ratio to shift towards reduction. This prevents ubiquinol from accepting electrons from any of the donors in this isopotential group. This, in turn, forces reduced ubiquinol within the complex I CoQ domain to transfer electrons to O_2 generating O_2^- and generating a ubiquinone that can accept the donor's electron. The concept of the ETC reducing backwards towards complex I is referred to as reverse electron transport. This mechanism accounts for the contribution of each of these enzymes to superoxide production.

5.2.ROS chemistry and deleterious effects Superoxide is a direct product from the mitochondrial electron transport processes but is only one of the ROS. Superoxide, however, has low reactivity with most organic molecules but will readily interact with other molecules (Fe^{2+}, FeS clusters, NO^-, and another O_2^-) including as a substrate for dismutase enzymes generating H_2O_2. H_2O_2 is able to diffuse across biological membranes or use aquaporins to mobilize throughout the cell[235]. H_2O_2 can also interact with metal ions (Cu^+, Fe^{2+}) to generate a hydroxyl radical (-OH). -OH is the most reactive form of ROS and interacts with most organic molecules rapidly[236]. The reactive free radicals are capable of targeting cellular macromolecules and interacting to cause damage using hydrogen abstraction reactions.

Lipid peroxidation is an example of such a reaction that targets unsaturated fatty acids generating a peroxyl-fatty acid radical. This radical can oxidize using another fatty acid to become a fatty acid peroxide and another fatty acid radical which can propogate the reaction.

This can lead to a series of fatty acid modifications in a region such as a membrane thus causing negative consequences to ion channels, membrane enzymes, transport proteins and altering the composition of the lipid bilayer[236].

Protein damage results from the interactions of free radicals with specific amino acid residues. Specifically, sulfhydryl groups are at risk in cysteine and methionine residues of becoming sulfoxides. Ring cleavage is possible in histidine and tryptophan residues. Other residues may form aldehyde or ketone carbonyls. All of these changes can alter structure and/or function of the peptide molecule. This will generally result in alter function of the protein and eventual proteolysis through the proteasome or lysosomal degradation. ROS can also directly interact with protein post-translational modifications to stabilize or destabilize specific signaling pathways as seen with ROS-induced stabilization of HIF-1α in the response to hypoxia. The proteolytic processes may be overwhelmed generating an abundance of dysfunctional protein aggregates[237–244].

Carbohydrates and sugars can interact with free radicals and amino acids to produce advanced glycation end products. These are amino acids that have been irreversibly altered by a carbohydrate to the extent that they cannot enter the 20S proteasome for degradation[245].

RNAs, which are already unstable in nature, can be oxidized by free radicals and truncated altering protein synthesis and resulting in abnormal protein translation[246,247].

DNA can be oxidized by ROS at both the base and the deoxyribose sugar[248,249]. The cell generally maintains a robust DNA damage/repair response but this too can become overwhelmed in situations of chronic, excessive ROS production[250,251]. nDNA oxidation has specific interest to those interested in radiation induced cancer generation but is of interest here because of the quantity of mitochondrial ROS produced in the ETC and the proximity to mtDNA for mutations.

ROS are able to react with DNA rapidly. Deoxyribose is bound to two phosphate at the 3' and 5' carbon. Each of these interact with an oxygen molecule on the $PO_3\gamma$. The interacting oxygen (from either the 5' or the 3' phosphate) can be oxidized by the free radical thus causing a single strand break[252,253]. Additionally, the DNA bases can be altered biochemically by ROS. Thymine can be reduced to form an enol base thymine-glycol that interferes with DNApolymerase. Guanine and adenine can be hydroxylated creating lesions in DNA. Other DNA oxidation products include 2-hydroxyadenine, 5,8-dihydroxycytosine, and 5-hydroxymethyluracil. At least 24 known alterations to DNA bases have been identified as products of ROS interactions. Each of these can cause mutations when replicated or inhibit transcription/replication unless repair mechanisms can compensate. Some mutations such as the 8-hydroxyguanine has no known repair mechanism because it is recognized as a different base[254–258].

5.3.**Mitigation of ROS** Free radicals are constantly generated by the cell and especially within the mitochondria but the damage that is associated with free radicals is generally mitigated by the cell in a series of mechanisms responsible for ROS homeostasis. Before ROS is even produced, uncoupling proteins embedded within the IMM are able to help control $\Delta\psi_m$ in such a way as to prevent ETC reduction and ROS formation[259]. However, once O_2^- is formed, the antioxidant system must come into play. The primary proteins involved in the antioxidant response are: 1) superoxide dismutase enzymes (SODs) which convert superoxide into hydrogen peroxide, 2) catalase which converts hydrogen peroxides into water, and 3) glutathione peroxidases which neutralize hydrogen peroxide by using reducing equivalents from reduced glutathione. Cytochrome C is a basic electron acceptor within the IMM and transfers electrons from complex III to complex IV of the ETS. Antioxidant activity has been demonstrated in Cytochrome C of isolated mitochondria and this can further contribute to proton pumping for

proton gradient and ATP production[260–264]. An isoform of SOD, SOD2 is specifically localized to the mitochondrial matrix for ROS detoxification[265–269]. While the family of SOD proteins does mitigate superoxide effectively, H_2O_2 is still quite toxic and can be mitigated by catalase. Catalase aids in the removal of H_2O_2 by producing molecular O_2 and H_2O though its contribution to overall cellular ROS mitigation has been a point of debate[270–272]. Glutathione is a tripeptide that is able to utilize a cysteine residue thiol group to accept an electron and become reduced. While multiple mechanisms exist to replenish the pool of oxidized glutathione, the GPx family of proteins is able to oxidize glutathione and reduce H_2O_2 into H_2O[273,274]. The expression and activity of these enzymes can help to control the redox state and ROS protection of the cell[275].

6.**Discussion** In summary, this review has discussed: 1) various CVDs, prevalence, mortality rates, and economic costs, 2) biochemical energetics, 3) mitochondrial genetics, 4) electron transport, and 5) ROS production. It is clear how heart disease can be attributed to biochemical alterations which are directly connected to the mitochondria. When the mitochondria become stressed, electron transport can begin generating free radicals which cause a series of negative chemical interactions involving DNA mutations. The proximity of ROS production inside of the mitochondria can result it accumulated mtDNA mutations. Because each of the 13 proteins encoded by mtDNA are paramount for proper electron transport, mutations can further propagate stress on the mitochondria starting the cycle over.

6.1.Bioenergetics in heart failure During HF, carbon substrate utilization is altered in the myocardium. The myocardial metabolic phenotype generally refers to the substrate and metabolic preferences of the myocardium and is useful in comparing HF patient phenotypes to healthy controls. The myocardial metabolic preferences are under the transient control of circulating glucose, lactate, fatty acids, insulin, catecholamines, and oxygen concentrations as

well as hemodynamics and inotropic state. The healthy heart generates the majority of energy from fatty acids that seem to be maintained during earlier stages of HF; however, fatty acid oxidation declines during end stage heart failure and shifts towards glycolytic substrates[276–279]. This is complicated, however, by age- and obesity-associated alterations in plasma FFA concentrations and insulin sensitivity. While this makes one associate greater fatty acid oxidation with healthy myocardia, cardiac lipotoxicity can occur by accumulation of triglycerides and ceramides causing increased apoptosis and contractile decline[280]. This situation is further complicated by an obesity paradox wherein obese patients are at greater risk to develop HF but once diagnosed, obesity is associated with greater mortality compared to lean HF patients especially following heart surgery[281,282]. This might be partly explained by other comorbidities in lean HF patients, specifically cachexia or wasting syndromes which would categorize one as lean but is associated with greatly reduced mortality.

6.2.Mitochondrial quality in heart disease Each of these processes, when working in concert, can contribute to favorable mitochondrial quality. In cardiac disease, multiple dysfunctional characteristics of mitochondrial quality are involved.

6.2.1.Dynamism and the heart Multiple studies have demonstrated that when balancing fission/fusion of mitochondria, development of large, elongated organelles seems beneficial when compared to small, fragmented mitochondria. In the heart, research demonstrates that ischemic injury causes increased fission and fragmentation of mitochondria and that genetically preventing fission to maintain a large, interconnected network improved cardiac outcomes[283–286]. But the case for fusion always being good and fission always being bad is more complicated, however. Genetically enhancing mitochondrial fission through Drp1 does not negatively affect cardiomyocyte function. Further, evidence in *Drosophila melanogaster* demonstrates that, while

fission can remove damaged mitochondrial components, fusion can 'poison' the reticulum with damaged components[287]. This is of severe detriment because the quick accumulation of mutated mtDNA products that are not selectively removed and destroyed but rather propagate and create mutated daughter organelles.

6.2.2.Mitophagy The process of mitophagy is inherently involved with mitochondrial dynamics because the portion of mitochondria to be destroyed must first be separated. Deletion of PINK1 results in adolescent onset cardiomyopathy. While assessments of mitophagy were not done, decreased respiratory function, overall mitochondrial quality and enlarged mitochondria were seen[287,288]. Deletion of Parkin also increased the hearts sensitivity to MI and leads to accumulation of damaged mitochondria with greater mtDNA mutations[289,290]. Similarly, genetic ablation of BNIP3 or Nix lead to cardiac hypertrophy through decreased cardiac function and abnormal mitochondria[291,292]. Overall, it would appear that too little or too much mitophagy leads to heart disease so the key questions that remain involve the balance and timing of mitophagic events. Genetically enhancing or limiting mitophagy over the lifespan of an organism both lead to chronic heart failure. Specifically, chronic overactivation seems to deplete the pool of healthy mitochondria contributing to oxidative deterioration while chronic lack of mitophagy allows damaged organelles to accumulate and propagate. Following acute stressors, however, such as MI or I/R, greater mitophagy allows enhanced clearance of damaged mitochondria to aid in recovery[293].

6.2.3.Biogenesis in heart disease If mitochondria are removed after ischemic damage, then the pool of oxidative organelles must be replenished through biogenesis. Unfortunately, the shift towards a 'fetal gene program' as seen in pathological cardiomyocytes decreases expression of genes involved in fatty acid metabolism including PPARs and PGC-1α[294]. The activity of the

TCA cycle as well as the contribution of β-oxidation-derived acetyl-CoA decreases at the end stages of heart failure[295]. This could be an end result of decreased transcriptional machinery for fatty acid oxidative. Studies have demonstrated that dilated cardiomyopathy is associated with decreased expression of PGC-1α and decreased mtDNA copy number (a surrogate for the amount of mitochondria) but no other markers for mitochondrial biogenesis were assessed[296]. Some evidence has emerged suggesting a potential cardioprotective effect of PGC-1α but more research is required to support these conclusions[297]. Others have reported impaired PGC-1α expression during heart disease but no causal results were demonstrated[298,299]. However, on the contrary, some approaches have demonstrated that PGC-1α can be detrimental to cardiac function during disease[300]. These studies demonstrate the need for further assessment of the coordination of mitochondrial biogenesis during heart disease.

6.3.Mitochondrial genetics and heart disease Multiple studies have shown a clear presence of heart disease (specifically cardiomyopathy, dilated cardiomyopathy, and fatal infantile cardiomyopathy) in patients with point mutations throughout the mitochondrial genome[301–303]. These studies did not establish a causal relationship, however. It was also shown that in humans with cardiomyopathies, gene expression of mitochondrial transcripts was elevated, perhaps to compensate for inefficiencies within the OXPHOS system[304]. Overexpression of TFAM or Twinkle can protect mtDNA, increase mtDNA copy number and contribute to cardioprotection though it is unclear if this is due to replication or transcription mechanisms[122]. Some polymorphisms in the TFAM gene have also been linked to early MI[305]. Wang et al.[306] demonstrate that conditional TFAM knockout leads to early onset heart failure and dilated cardiomyopathy suggesting that the mitochondrial gene expression machinery is requisite post-natally. Specific mtDNA mutations are highly associated with a variety of disease processes. The

majority of diseases with mtDNA mutation are due to tRNA mutations likely because they are so prevalent in the small mitochondrial genome and they will negatively affect each protein-encoding gene[213]. Mutations in mtDNA over the lifespan can also contribute to worsening MI or I/R damage to cardiomyocytes[307]. In summary, it is clearly evident that mtDNA expression is vital to cardiac health through the use of the mtDNA-encoded OXPHOS proteins. Any alterations in global mtDNA expression (transcription/replication), global mtDNA mutations (tRNAs), or specific protein mutations (any of the thirteen mtDNA-encoded proteins) can cause or worsen heart disease and stress.

6.4.Electron transport and heart disease Studies have shown a clear presence of heart disease (specifically cardiomyopathy, dilated cardiomyopathy, and fatal infantile cardiomyopathy) in patients with point mutations of ETC complexes I, III, IV, and V as well as tRNA mutations and large DNAa mutations[301–303]. However, these studies did not establish a causal relationship. Inhibition of electron movement can help reduce components of the ETS during ischemia and prevent reperfusion induced myocardial damage[308,309]. These results led to multiple studies attempting to identify which complex of the ETC contributes to mitochondrial damage during ischemia. Results have outlined that blockade of Complex I and III but not IV protect ischemic injury. It is puzzling though because the authors suggest the ischemic injury is from some point distal to complex III but proximal to complex IV with the only intermediary being cytochrome C, a simple electron carrier[310–313].

6.5.ROS and heart disease Under normal physiological conditions, the amount of ROS produced by the cell is easily managed by ROS mitigation enzymes. During pathological conditions such as chronic cardiomyopathies, alterations in normal electron transport function can cause substantial increases in ROS production and cause cellular damage. Acutely, during

ischemic episodes on the heart, the reintroduction of blood supply does not restore the hypoxic organ but rather causes a reperfusion injury increasing substantial amounts of ROS, inflammation, and apoptosis[314]. Indeed, aging related mutations in mtDNA contribute to cardiomyopathy and this can be mitigated by overexpression of catalase[315]. Mice lacking the gene for MnSOD are perinatally lethal due to advanced cardiomyopathy[269]. Even in young drug users, cocaine-induced cardiomyopathies have been attributed increased oxidative stress through impaired redox handling in the heart[316]. Current research is focusing on mitochondrion-permiable antioxidants to help mitigate ROS damage, especially following ischemia[317,318]. While ROS can act as a normal cell signaling molecule when controlled, the consensus of literature suggests that overactive ROS production and impaired ROS handling are characteristics and causes of heart disease[319].

6.6.Conclusion In conclusion, the process of mt-mRNA translation is 1) vital to generate ETC components is 2) indispensable for proper OXPHOS within the mitochondrion, 3) susceptible to negative effects by ROS and mtDNA mutations, 4) can contribute to the oxidative capacity and redox state of the cell and thus must be highly involved in heart disease. This leads to the development of the following research aims:

Specific Aim I: Hypertrophic heart disease induced by aging and obesity alters the machinery for mt-mRNA translation. Major cardiovascular disease is the leading cause of death in the United States and is linked to obesity and aging. Chronic heart failure is associated with fibrosis, systolic impairments, and reduced oxidative capacity leading to increased ROS production. The reductions in oxidative capacity have been explained, in part, by decreased electron transport through complexes I and IV of the ETC which contain a majority of subunits

encoded by mtDNA and are created by mt-mRNA translation machinery. For experiments in this aim, we will use a murine model of aging combined with obesity to induce cardiac hypertrophy. Using primarily immunoblot, we will test if deteriorations in protein content of mt-mRNA translation machinery could explain increases in ROS production and oxidative capacity seen in cardiac muscle of aged, obese mice.

Specific Aim II: Pathophysiological cardiac atrophy is demonstrated with impaired mt-mRNA translation machinery and shown to contribute to reduced oxidative capacity. Recently, evidence has emerged of an atrophic state seen in the cardiac musculature of cancer patients. This decrease in the myocardium has been associated with a decreased rate of protein synthesis and an increase in protein degradation signaling. While a decrease in ventricular size may be responsible for the relative increased incidence of heart failure among cancer patients, the mitochondrial oxidative state and ROS mitigation may play a critical role. Using LLC tumor implantation in mice, we will induce cardiac atrophy and use immunoblot procedures to determine if mitochondrial content, ROS mitigation, and mt-mRNA translation machinery are altered in a mouse model cancer-induced cardiac atrophy.

Specific Aim III: mt-mRNA translation initiation is required for maintenance of oxidative potential and oxidation-reduction state. Oxidative capacity requires appropriate mitochondrial ETC components which are necessary for appropriate oxidative phosphorylation without excess production of ROS. While chronic heart disease and the associated production of ROS is partly due to reduced capacity for ROS mitigation, the potential exists for reduced efficiency of mt-mRNA translation to result in both impaired oxidative capacity and increased production of ROS, simultaneously. Using cardiomyocytes in vitro, we will genetically inhibit mtIF2 mRNA and examine metabolic oxidative flux and redox characteristics. These experiments will help

determine how mtIF2 contributes to mt-mRNA translation as a candidate protein responsible for the detriments in ETC components, subsequent decrements in oxidative capacity and redox state of cardiac muscle cells.

These experiments will demonstrate that mt-mRNA translation is required for oxidative metabolism and control of harmful ROS production. I have compiled these aims into two manuscripts based on the congruency of the results. Experiments from Aim II are presented in manuscript 1 entitled, “Cancer-induced Cardiac Atrophy Adversely Affects Myocardial Redox State and Mitochondrial Oxidative Capacity”. Aims I and III have been combined in manuscript 2 entitled, “Mitochondrial Translation Initiation Factor 2 is Necessary for Cardiac Oxidative Capacity as Evident during Age-induced Cardiac Hypertrophy”.

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Chapter 3 – Manuscript 1

Title: Cancer-induced Cardiac Atrophy Adversely Affects Myocardial Redox State and Mitochondrial Oxidative Capacity

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Running Title: Metabolic Cardiomyopathy during Cancer Cachexia

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Abstract: Cachexia presents in nearly 80% of advanced cancer patients; however, cardiac atrophy in cachectic patients has received little attention. This unique form of cardiomyopathy in cancer patients contributes to increased occurrence of myocardial ischemia and adverse cardiac events compared to age-matched population norms. Research on cardiac atrophy has focused on cardiac remodeling; however, alterations in oxidative metabolic properties may be a significant contributor to this form of cardiac disease. **Purpose:** To determine how cancer-induced cardiac atrophy alters *in-vivo* mitochondrial turnover, mitochondrial mRNA translation machinery, ROS scavenging and *in-vitro* oxidative characteristics. **Methods**: PBS or Lewis lung carcinoma (LLC) tumors were implanted in mice and grown for 28 days to induce cardiac atrophy. Hearts were examined for endogenous optical metabolic species, and immunoblotted to assess markers of mitochondrial function. H9c2 cardiomyocytes were cultured in a control media or media collected from LLC cells in combination with a mitochondrially-targeted antioxidant (MitoTempo). Cells were analyzed for production of ROS, oxidative capacity, and resistance to hypoxic stress. **Results**: LLC hearts demonstrated ~15% lower optical redox ratio (FAD/FAD+NADH) indicating greater glycolytic reliance compared to PBS controls. When compared to PBS, LLC hearts showed ~50% greater mitochondrial content markers (COX-IV, VDAC) attributed to ~50% lower PINK1/Parkin-mediated mitophagy markers while mitochondrial protein synthesis and biogenic proteins PGC-1α, PPARα, and PPARδ were not different. Mitochondrial mRNA translation machinery was unchanged between groups relative to amount of mitochondria. Mitochondrial DNA-encoded CytB was ~30% lower in LLC hearts suggesting impairments in outcomes of mitochondrial mRNA translation. ROS scavengers GPx-3 and GPx-7 were ~50% lower in LLC hearts. Treatment of cardiomyocytes with LLC-conditioned media resulted in higher ROS (25%), lower oxygen consumption rates (10% at

basal, 75% at maximal), and greater susceptibility to hypoxic-insult by ~25% -- all of which were reversed by addition of MitoTempo. **Conclusion:** These results substantiate metabolic cardiotoxic effects attributable to tumor-associated factors and provide new insight into interactions between mitochondrial mRNA translation, ROS mitigation, oxidative capacity and hypoxia resistance.

Graphical Abstract:

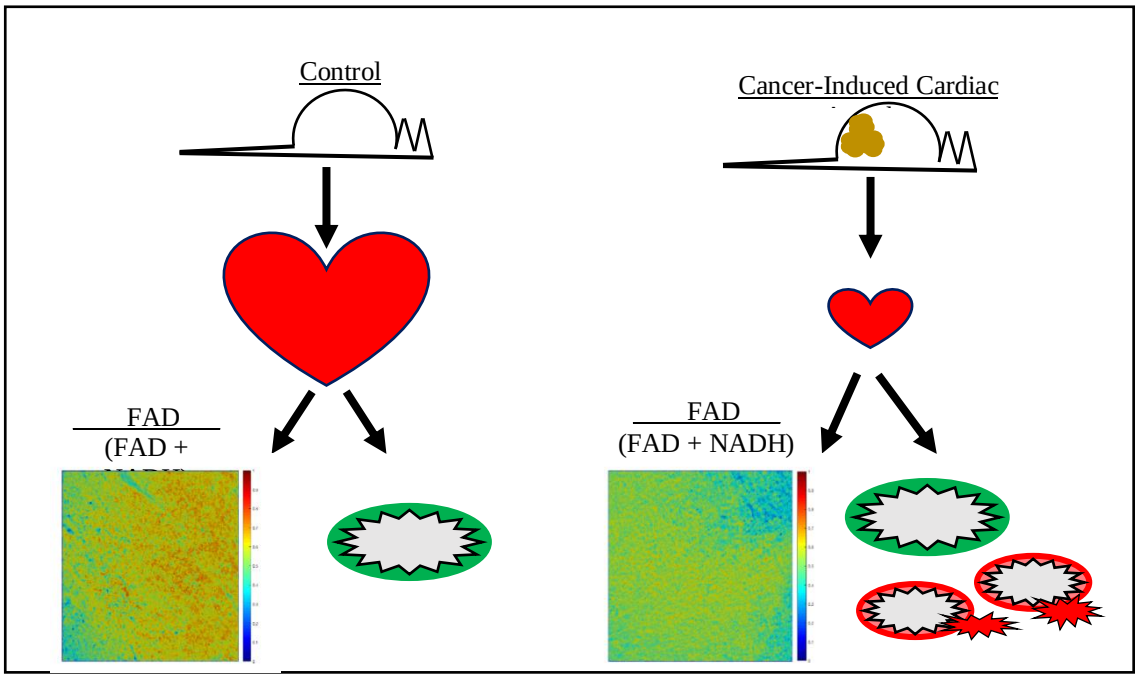

Figure: Graphical abstract outlining the key findings of this publication.

Highlights:

- Cancer leads to altered metabolism and atrophy of the heart
- Mitochondrial turnover, mRNA translation, and ROS scavenging are all affected
- Mitochondrial ROS scavengers protect aerobic capacity and hypoxia resistance
- Antioxidant drugs mitigate the effects of tumor-associated changes to the heart

Keywords: Cardiac cachexia, optical redox imaging, mitochondrial translation, cardiac wasting, cardio-oncology, mitochondrial antioxidant

[*] Abbreviations: COX-IV – cytochrome oxidase subunit IV, CytB – mitochondrial-encoded Cytochrome B1, FAD – Flavin adenine dinucleotide, GPx – Glutathione peroxidase, HIF-1α – Hypoxia-inducible factor 1α, LLC – Lewis Lung Carcinoma, mtIF2/3 – mitochondrial translation initiation factors 2/3, mtDNA – mitochondrial-encoded DNA, mt-mRNA – mitochondrial-encoded mRNA, NADH – nicotinamide adenine dinucleotie, nDNA – nuclear encoded DNA, PBS – Phosphate-buffered saline, PGC-1α – PPARγ co-activator-1α, PPARα/δ – Peroxisome proliferator-activated receptor α/δ, OXPHOS – Oxidative phosphorylation system, Redox – oxidation/reduction, ROS – Reactive Oxygen Species, SOD – Superoxide dismutase, TACO1 – Translational co-activator of COX1, TFAM – mitochondrial transcription factor A, TPEF – Two-photon excitation fluorescence, VDAC – voltage-dependent anion channel

Introduction

Cancer cachexia is a progressive deterioration of functional capacity characterized predominantly by a loss of skeletal muscle mass with impacts on multiple other organs[1]. While cachexia is present in as many as 80% of advanced cancer patients[2], the comorbidity of cardiac atrophy in cachectic patients has received little attention[3]. The clinical focus in targeting the underlying malignancy often dismisses cardiotoxic effects of radiation and chemotherapeutic treatment approaches[4,5]. However, recent data demonstrates that advanced cancer contributes to detrimental cardiac alterations including reduced left ventricular systolic function and decreased cardiac musculature resulting from tumor-derived factors rather than from chemo/radiotherapies[6,7]. While the compounding effects of tumor-related cardiac alterations, treatment modalities, and potential of underlying heart disease make it difficult to determine the etiology of cardiac atrophy seen in cancer patients[5], further research is desperately needed to understand how cancer contributes to changes in the cardiac metabolic state.

Heart failure has been tightly linked to alterations in metabolic substrate utilization and detriments to mitochondrial oxidative capacity[8,9]. Specifically, patients with heart disease exhibit a shift from fatty acid oxidation towards greater reliance on glucose as a source for ATP[10]. Mitochondria have therefore become a key target in combatting this metabolic reprogramming in heart disease as these organelles contribute ~90% of ATP generated in the healthy myocardium[11–13]. Previous research supports these efforts to target mitochondria because electron transport through oxidative phosphorylation (OXPHOS) complexes I and III limits oxygen flux during left ventricular systolic dysfunction[14]. When electron transport at these specific OXPHOS sites decreases, they can become primary producers of reactive oxygen species (ROS)[15]. These free radicals, when left unmitigated[16], can contribute to DNA mutations

and damage, protein modifications, and membrane lipid oxidation[17–23]. Because of the proximity to mitochondrial DNA (mtDNA), ROS produced at OXPHOS complexes has the potential to mutate mtDNA and further contribute to detriments in OXPHOS electron transport seen in heart failure[24]. This interconnected relationship between mtDNA mutations, OXPHOS deficits, and ROS production provides the basis for Harman's free radical theory of aging[25] and more specifically, the mitochondrial theory of heart disease[26]. While progressive loss of mtDNA copy number, increased mtDNA mutations[27,28], OXPHOS deterioration[14], and excess ROS[29] are well established contributors to traditional cardiomyopathies, the mitochondrial alterations during cancer-induced cardiac atrophy remain unclear.

Key to elucidating mitochondrial bioenergetics during cancer-induced cardiac atrophy is understanding the processes of mitochondrial turnover and maintenance of OXPHOS complex activity. The balance of mitochondrial biogenesis and mitophagy contribute to fluctuations in organelle volume within the myocardium, however, mitochondrial mRNA(mt-mRNA) translation machinery likewise contributes to OXPHOS activity and proper electron transport[30–35]. A balance between mt-mRNA translation and cytoplasmic translation is required for proper assembly of OXPHOS complexes[36] and during situations of impaired mt-mRNA translation, metabolic side-effects are evident[37–39]. Improper translational insertion[40] by mt-mRNA translation machinery also contributes to ROS production and alters antioxidant defenses through the mitochondrial unfolded protein response[41,42]. The connection between mt-mRNA translation and cardiomyocyte oxidative capacity has not been well established[43,44].

In an effort to better understand the impact of cancer-induced cardiac atrophy on mitochondrial oxidative characteristics, the purpose of this investigation was to examine mitochondrial turnover and mt-mRNA translation alterations during this unique form of heart

disease. Furthermore, we investigated tumor-associated changes in antioxidant defense as a mechanism of excess ROS production and how this contributes to the deleterious effects on cardiac oxidative reserve capacity and resistance to hypoxia. By using an established, *in-vivo* model of cancer-induced cardiac atrophy we present label-free metabolic oxidation-reduction (redox) characteristics indicative of heart disease, alterations in mitochondrial content, mt-mRNA translation, and ROS scavengers. We further demonstrate the potential efficacy of mitochondrially-targeted antioxidants to mitigate excessive ROS production and how this approach protects against hypoxic insult to cardiomyocytes *in-vitro*.

Materials and Methods

Animal model of cancer-induced cardiac atrophy

All methods were approved by the Institutional Animal Care and Use Committee at the University of Arkansas. C57BL6/J mice were injected with $1x10^6$ Lewis Lung Carcinoma cells (LLC) or equal volume sterile phosphate-buffered saline (PBS) as previously described[45,46]. LLC cells were purchased from American Type Culture Collection (ATCC, CRL-1642) and grown in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) with media changed every second day. Cells were trypsinized, centrifuged, and counted prior to implantation. Tumors were allowed to grow for 28 days before mice were euthanized and hearts excised. Multiple small sections of the left ventricle were separated to allow protein and histological assessment of the same sample. Heart samples were snap frozen and stored at -80ºC before further analysis. Mice were injected with 99.9% ^2H_2O in the peritoneum (20µL/g body weight, Sigma-Aldrich, St. Louis, MO, USA) 24 hours prior to euthanasia. 4% ^2H_2O in H_2O (v/v) was provided as drinking water ad libitum for the entire 24 hour period as previously described[47].

Two-photon excitation fluorescence of endogenous NADH and FAD

Two-photon excitation fluorescence (TPEF) was used to measure endogenous fluorescence of NADH and FAD as previously described[48]. Briefly, laser excitation by MaiTai Ti:Sapphire laser source (Spectra-Physics, Santa Clara CA) was tuned to 755nm or 860nm. Images were acquired using a resonant-galvo scanner and GaAsP photomultipler tubes (H7422-40, Hamamatsu) with 460/40nm (NADH), 525/45nm (FAD), and 600/70nm (Rhodamine) filters. 10μm section of the left ventricle were cut along the short axis. Slides were imaged (512x512 pixels, 16bit depth, 260μm^2) and the pixel-wise FAD/(NADH+FAD) ratio normalized to rhodamine was calculated. Multiple fields were imaged for each sample and averaged to represent an individual biological sample. NADH and FAD image analysis was completed using MATLAB (MathWorks, Natick, MA).

Immunoblot analysis

Protein was extracted from small fractions of muscle taken directly from the left ventricle, separated by 8% or 12% SDS-PAGE and transferred to polyvinylidene fluoride membrane as described[49,50] alongside a molecular weight ladder. Membranes were blocked using 5% milk in TBS (w/v) with 0.2% tween and incubated with specific primary antibodies at 4°C overnight. Primary antibodies were specific to HIF-1α (NB100-105, Novus Biologicals), COX-IV (Cell Signaling 4855S) VDAC (Cell Signaling, 4866S), PGC-1α (Santa Cruz sc-13067), PPARα (Santa Cruz sc-9000), PPARδ (Santa Cruz sc-7197), BNIP3 (Cell Signaling 3769), PINK-1 (Santa Cruz sc-33796), Parkin (Cell Signaling 42115), pSer65-Parkin (Abcam ab154995), mtIF2 (Santa Cruz sc-365477), mtIF3 (Origene TA800421), TACO1 (Abcam ab121688), CytB (Santa Cruz 11436), Total OXPHOS antibody cocktail (Abcam ab110413, Complex I – NDUFB8, Complex II – SDHB, Complex III – UQCRC2, Complex IV – MTCO1,

Complex V – ATP5A), SOD1 (Genetex GTX100554), SOD2 (Cell Signaling 131945), SOD3 (R and D Systems AF4817), GPx7 (Genetex GTX117516), GPx3 (Genetex GTX89142), and Catalase (Cell Signaling 140975). Using appropriate HRP-conjugated or fluorescent conjugated secondary antibodies, protein antigens were visualized within a linear range by either ECL on Protein Simple FluorChemM (Minneapolis, MN) or near-infrared fluorescence on Odyssey Fc (LI-COR, Lincoln, NE) and analyzed using ImageStudio Software (LI-COR). Bands were normalized to the 45kDa Actin band of PonceauS as loading control. For each experiment, all groups were represented on each membrane and normalized to control.

Mitochondrial Isolation and 24-hour Protein Synthesis

Isolation of mitochondrial subpopulations and fractional synthesis rates were determined from samples as previously described (34). Briefly, 30 mg of left ventricle was homogenized isolation buffer 1 (10 mM HEPES, 20 mM sucrose, 50 mN mannitol, 2mM EDTA, 0.25% v/v protease inhibitor cocktail, pH 7.4) and centrifuged at 650 x G. The supernatant was then further centrifuged at 10,000 x G for 10 minutes and pellet collected for analysis of subsarcolemmal (SS) mitochondria. SS mitochondria were washed in isolation buffer 2 (50 mM HEPES, 5mM EGTA, 1mM ATP, 100mMKCL, 5 mM $MgSO_4$, 0.25% v/v protease inhibitor cocktail, pH 7.4) The intermyofibrillar (IMF) mitochondria were separated from the myofibrils in pellet created in the first centrifugation first by dounce homogenization in isolation buffer 3 (100 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM Beta-glycerophosphate, 1.5% w/v BSA, 0.25% v/v protease inhibitor cocktail, pH 7.5). The resulting homogenate was centrifuged at 650 x G and the resulting supernatant was centrifuged at 10,000 x G for 10 minutes and mitochondrial-rich pellet collected. SS and IMF proteins were hydrolyzed into individual amino acids by heating for 24 hours at 100°C in 6N HCl. An aliquot of the hydrolysate was dried down and

mixed with a 3:2:1 solution of methyl-8, methanol, and acetonitrile to determine ^{2}H-labeling of alanine on its methyl-8 derivative. The solution was then placed in a GC-MS and analyzed for the ratio of labeled alanine to unlabeled alanine was used to calculate protein synthesis. In order to normalize results based on the precursor pool of $^{2}H_2O$, plasma was reacted with 10 M NaOH and a 5% solution of acetone in acetonitrile for 24 h in order to conjugate the free $^{2}H_2O$ to acetone. The solution was extracted by adding Na_2SO_4 and chloroform, and analyzed on the GCMS. FSR of proteins was calculated using the equation EA × [EBW × 3.7 × t (h)]−1 × 100, where EA represents amount of protein-bound [^{2}H]alanine (mole% excess), EBW is the quantity of $^{2}H_2O$ in body water (mole% excess), 3.7 represents the exchange of ^{2}H between body water and alanine (3.7 of 4 carbon-bound hydrogens of alanine exchange with water) and t(h) represents the time the label was present in hours.

H9c2 Culture experiments

H9c2 ventricular cardiomyocytes were purchased from ATCC (CRL-1446) and grown at $37^{0}C$, 5%CO_2, and 20% O_2 in DMEM containing 10% FBS and 1% pen/strep (GM) and changed every second day. When cells were ~75% confluent, $2x10^{4}$ cells/well were sub-cultured in 96-well plates for 24 hours before media was replaced with control media (CM; DMEM containing 2.5% FBS, 1% pen/strep) or LLC-conditioned media (LCM) as previously described[51,52]. To generate LCM, LLC growth media was collected after two days of incubation in $162cm^2$ flask with LLC density ending ~75% confluence. Media was centrifuged and filtered to remove cells and cell debris and diluted 1:4 (v/v) with serum-free DMEM. MitoTEMPO (MitoT; SML0737, Sigma-Aldrich, St. Louis, MO) diluted in PBS was added to CM or LCM at a concentration of 2 μM. After 2 hours incubation in respective media, 5μM MitoSOX Red (M36008, Invitrogen) in PBS was added to cells for 10 minutes, rinsed, and cells were visualized at 510/580nm (ex/em)

on Nikon TiS epifluorescent microscope (Melville, NY) to assess mitochondrial superoxide production.

Bioenergetic flux analysis

Oxygen consumption rates (OCR) were analyzed using Seahorse XFp extracellular flux analyzer (Agilent, Santa Clara, CA) according to manufacturer instructions and as previously described[53]. Briefly, $2x10^4$ cells were seeded per well in GM. After 24 hours, media was removed, cells were rinsed with sterile PBS, and replaced with CM or LCM with or without 2 μM MitoT. After 24 hours incubation, media was removed and replaced with Seahorse Assay media containing 7 mM glucose, 2 mM pyruvate, and 2 mM glutamine. OCR was measured prior to and following sequential addition of 1 μM oligomycin, 1 μM FCCP, and 1 μM rotenone/Antimycin A. This allowed assessment of cellular OCR related to basal respiration and maximal uncoupled respiration. Reserve respiration was determined as the difference between maximal and basal after normalizing to non-mitochondrial oxygen consumption.

Hypoxic exposure and MTT viability assessment

In order to assess the resistance of H9c2 cells to hypoxic challenge, $2x10^4$ cells/well were plated in 96-well plate and incubated in GM for 24 hours. Media was replaced with CM or LCM with or without 2 μM MitoT. After 24 hours incubation at 5% CO_2 and 20% O_2, media was replaced with serum-free DMEM and cells were placed in a dual gas controlled (Oxycycler C42, Biospherix, Parish, NY) incubator sub-chamber. Oxygen was flushed by nitrogen and maintained at 1% O_2 and 5% CO_2 for 6 hours. The combination of serum-free media with a hypoxic environment was used to simulate ischemic conditions[54]. Following hypoxic exposure, 1 mM MTT (M6494, Invitrogen) was added to cells and incubated ~21% O_2 for 2 hours. The resulting formazan crystals were solubilized by addition of 100 μL of 350 mM SDS in 0.01% HCl and

absorbance was read at 570 nm. A separate plate was maintained at ~21% O_2 to serve as 100% viability control.

Statistical Analysis

Statistics were calculated and visualized using GraphPad Prism v6.0. To compare PBS to LLC groups, Student's t-test was used with α set at 0.05. For cell culture experiments, a 2x2 ANOVA was used to compare main effects or interactions between groups (CM x LCM vs. Control x MitoT). Where significant omnibus differences occurred, Tukey's post-hoc analysis was used to investigate changes within groups. Cell culture experiments were analyzed using 3 biological replicates and at least 3 technical replicates. Data presented represent mean ± SEM.

Results and Discussion

Cancer cachexia contributes to cardiac atrophy and altered optical metabolic properties

Four weeks of LLC tumor implantation resulted in severe skeletal muscle and fat mass loss including a ~30% reduction in muscle cross-sectional area as we previously reported in the same animals demonstrating cancer cachexia in this model[45,46]. In the current study, we used the same animal cohort to analyze cardiac alterations associated with tumor implantation[45,46]. Total wet weight of the heart was 10% lower in LLC compared to PBS demonstrating atrophy of the myocardium ($p < 0.01$, Table 1). Tibia lengths were not different between experimental conditions suggesting body size was similar between groups so we have presented raw heart mass.

To further characterize the metabolic alterations associated with this form of cardiac atrophy, we performed TPEF of endogenous FAD and NADH. This approach has demonstrated to be a powerful, label-free assessment of metabolic characteristics in a variety of cell and tissue types[53,55–58]. TPEF is a powerful new technique to identify metabolic intermediates in a label-free

assessment of cellular metabolic state[59]. In calculating the optical redox ratio, we identified a significantly lower redox ratio in LLC heart samples compared to PBS (Figure 1A, B; mean of 0.75 in PBS vs. 0.68 in LLC, $p < 0.01$). A lower optical redox ratio is indicative of less utilization of mitochondrial oxidative metabolism and greater reliance on glucose[56]. Additionally, immunoblot analysis of HIF-1α indicates ~100% greater content in LLC compared to PBS (Figure 1C). HIF-1α is a key sensor of oxygen levels and can downregulate mitochondrial oxidative metabolism while promoting glycolytic enzymes; a characteristic of cardiomyopathy metabolic changes[60,61]. These results extend upon previous evidence of showing decreased heart size in colorectal cancer-induced cardiac atrophy[62] and the shift away from β-oxidation towards glycolysis seen in heart failure[8].

Cardiac atrophy disrupts mitochondrial clearance

Mitochondrial function has been a target for heart failure treatments to combat against the metabolic shift that occurs[13]; therefore, we analyzed proteins associated with mitochondrial content and biogenesis. Both COX-IV and VDAC levels were significantly greater in LLC compared to PBS by ~40% and ~75%, respectively (Figure 2B, $p < 0.05$) indicating elevated mitochondrial content in LLC hearts. There were no differences measured between experimental groups in subsarcolemmal or intermyofibrillar mitochondria fractional synthetic rates nor in protein content of regulators of mitochondrial biogenesis including PGC-1α, PPARα, or PPARδ (Figure 2B,C; $p > 0.05$). In other models of heart failure, downregulation of proteins associated with lipid metabolism including PPARα and PGC-1α as well as respiratory-chain complex activity has been reported[63]. However, electron microscopy reveals that the amount of mitochondria are increased in many forms of cardiomyopathy despite other structural malformations[64]. Greater mitochondrial content (COX-IV, VDAC) absent upregulation of

mitochondrial biogenesis (i.e. mitochondrial FSR, PPARs, PGC-1α) suggests alternate processes contributing to the overall mitochondria pool. Total mitochondrial content is a highly regulated balance between biogenesis and mitochondrial-specific autophagy (mitophagy)[30], thus an imbalance in mitochondrial content between PBS and LLC with no alteration in biogenesis predicates mitophagy as a contributing factor. This led us to examine markers of two common mitophagic pathways, BNIP3 and PINK1/Parkin. Protein content of BNIP3 was unchanged between PBS and LLC hearts ($p > 0.05$) while the total content of PINK1 and the ratio of phosphorylated to total Parkin were both decreased by ~50% (Figure 3; $p < 0.05$). These markers indicate PINK1/Parkin mediated mitophagy detriments may contribute to a build-up of (presumably) defective mitochondria which has, as yet, remained unclear during chronic heart disease[31]. Parkin-deficient mouse models present with accumulation of depolarized mitochondria following myocardial infarction indicating the importance of this pathway in the clearance of these damaged organelles in the myocardium[65]. These results suggest an important role for Parkin-dependent mitochondrial clearance in cancer-induced cardiomyopathies which could result in accumulation of depolarized mitochondria. The consequences of depolarized mitochondria that are not efficiently broken down in the myocardium remains uncertain.

LLC myocardial mt-mRNA translation machinery is unable to maintain mtDNA-encoded OXPHOS subunits

mt-mRNA translation machinery allows proper translation of 13 protein subunits encoded by mt-DNA which are required for proper production of core portions of OXPHOS complexes[34]. TFAM is a key factor involved in mtDNA transcription[66] and the primary controllers of mt-mRNA translation are two mammalian mitochondrial initiation factors mtIF2 and mtIF3[67]. Additionally, co-translational insertion using translational coactivators – such as TACO1 in

mammals – is required for proper assembly of OXPHOS complexes[40]. These factors work to create a balance between mt-mRNA translation and cytoplasmic translation to produce and assemble OXPHOS complexes in a manner that optimizes electron transport and ATP production[36]. TFAM, mtIF2, mtIF3, and TACO1 protein contents were all significantly higher in LLC compared to PBS by ~50-125% ($p < 0.05$). However, the mt-mRNA translation product CytB was not significantly altered by the LLC tumor (Figure 4A; $p > 0.05$). To assess content of proteins relative to the total mitochondrial pool, we normalized mt-mRNA translation proteins to VDAC protein. We observed no differences seen in TFAM, mtIF2, mtIF3, or TACO1 between experimental groups ($p > 0.05$); however, CytB, when normalized to VDAC as a marker of mitochondrial content, was ~45% lower in LLC compared to PBS (Figure 4B; $p < 0.05$). When complex content was assessed using immunoblot analysis of individual core subunits, we found no significant difference between groups for Complex I, III, or V ($p > 0.05$) but found significantly elevated content of Complexes II and III (Figure 4C ,$p < 0.05$). The proteins encoded by mtDNA are integral, core subunits in OXPHOS complexes I, III, IV, and V[34]. In each complex, the mtDNA-encoded channels are vital for appropriate electron transport or proton translocation across the membrane[68]. Where mutations or alterations in expression of mtDNA-encoded proteins arise, oxidative complications associated with cardiac illness are evident[19,24]. The results presented here suggest that during this form of cardiomyopathy, the machinery responsible for expression of mtDNA-encoded transcripts is maintained relative to the mitochondrial content as measured by VDAC. However, resulting mtDNA-encoded protein subunits of OXPHOS may not be equally maintained suggesting other possible issues responsible for deficient mitochondria-encoded protein expression. By assessing amount of complex formation through immunoblot of subunits that are labile when not assembled, we

found that complexes with a large proportion of mtDNA-encoded proteins were no different between groups (Complex I, IV, V) while those with primarily nDNA-encoded portions (Complex II, III) were elevated concomitant with mitochondrial content markers. This reduced content of mtDNA-encoded proteins can result in misincorporation of OXPHOS subunits and prevent efficient electron flow through complexes I and III and result in greater production of superoxide, presumably through reverse electron transport[69]. Mitigation of free radicals by antioxidant enzymes may present as a potential mechanism to protect against excessive ROS produced during heart disease[29].

LLC contributes to altered ROS scavenger protein levels

In an effort to better understand handling of ROS, we assessed various proteins involved in superoxide (superoxide dismutases: SOD1, 2, 3) and hydrogen peroxide dissipation (Catalase, Glutathione Peroxidases: GPx-3,7). No differences in protein content of SOD-1, 2, or 3 were found between PBS and LLC hearts (Figure 5A; $p > 0.05$). Protein content of Catalase was 80% more abundant in LLC animals compared to PBS ($p < 0.05$), while GPx-3 and GPx-7 were both significantly lower in LLC compared to PBS by ~60% ($p < 0.01$) and ~50% (Figure 5B; $p < 0.05$), respectively. Taken together, these results suggest no alterations in the control of superoxide radicals through dismutase proteins. However, alterations appear in proteins involved in hydrogen peroxide (H_2O_2) clearance. Specifically, the elevation seen in Catalase content was unexpected because, previously, elevated Catalase has been linked to mitigation of age-dependent heart disease in mice[70]. The levels of GPx-3 and -7 were both reduced by the tumor-associated cardiomyopathy. Taken together, this could signify an overall decrease in ability to handle H_2O_2 because GPx's maintain greater affinity for[71] and reactivity with H_2O_2 compared to catalase at physiologically relevant concentrations[72]. Excessive H_2O_2 has been seen in aging-

induced heart disease despite greater activity of Catalase[73], which may contribute to cardiac cell death[74] and reduced mitochondrial oxygen consumption[75]. While direct measurements of ROS production and OCR were not assessed in PBS and LLC hearts, we next sought to determine how tumor-associated factors could alter mitochondrial ROS production and OCR in cardiac cells *in-vitro*.

Mitochondrial antioxidants mitigate effects of tumor-associated factors on cardiomyocyte ROS production and oxidative capacity

To directly assess impacts of tumor-associated factors on the myocardium, independent of circulating factors (immune cells, compliment), cardiomyocytes were cultured in CM or LCM alone or in combination with MitoT, a mitochondria-targeted antioxidant. Two hours following treatment, ROS accumulation was assessed using MitoSOX fluorescence. H9c2 cardiomyocytes showed ~25% greater MitoSOX fluorescence when incubated in LCM alone ($p < 0.001$). The combination with MitoT had no effect on MitoSOX in CM-treated cells ($p > 0.05$), however, combined LCM and MitoT treatment showed significantly lower MitoSOX fluorescence compared to LCM alone ($p < 0.001$) to the point that LCM + MitoT was not different compared to CM (Figure 6A, B; $p > 0.05$). To further test the detrimental effects of LCM, cardiomyocytes were treated for 24 hours and oxygen flux analysis was assessed (Figure 6C). Basal OCR was ~15% lower following 24 hours of LCM treatment compared to CM ($p < 0.01$) with no effects of MitoT on LCM treated cells (Figure 6D; $p > 0.05$). When cells were treated with FCCP to simulate maximal OCR rates, LCM control cells had a maximal OCR that was ~1/3 that of the CM ($p < 0.01$). When combining LCM with MitoT, maximal OCR was higher than LCM control ($p < 0.01$) and not different from either CM treatment (Figure 6E; $p > 0.05$). Using these values, we were able to calculate the reserve OCR which followed a similar pattern of severe reduction

(~75% capacity) relative to CM following 24 hours LCM ($p < 0.01$) but not when combined with MitoT (Figure 6F; $p > 0.05$). These results demonstrate a clear connection between tumor-associated factors and alterations in mitochondrial metabolic characteristics in cardiac muscle cells. The initial increases in ROS production following LCM treatment can be mitigated using a mitochondrial antioxidant and overtime can result in functional rescue of mitochondrial oxidative reserve. One way myocardial oxidative capacity contributes to cardioprotection is through resistance to hypoxic-insult[76–78]. In order to test if our results in OCR reflected susceptibility to hypoxia-induced cell death, we exposed cells to 1% O_2 for 6 hours and assessed cell viability. All groups demonstrated significantly lower viability following hypoxia compared to normoxic control (dashed line) with LCM control being significantly lower compared to CM and LCM + MitoT cells by ~30% (Figure 7A; $p < 0.001$). These data suggest that cancer-induced oxidative alterations may be rescued through mitigation of mitochondrial ROS. There is considerable need to better characterize the oxidative metabolic properties of the myocardium undergoing cancer-induced structural alterations. These results substantiate cardiotoxic effects attributable to tumor-associated factors and provide new insight into interactions between ROS mitigation, cardiac oxidation and hypoxia resistance.

Our findings suggest cancer-induced cardiac atrophy presents with altered metabolic properties associated with heart disease including greater reliance on glycolysis assessed using a label free measurement of endogenous redox species and elevation in HIF-1α. We additionally present impaired mitochondrial clearance, disruptions in mt-encoded protein expression, and less protein involved in ROS mitigation. Finally, *in-vitro* experiments show that tumor-specific factors exacerbate cardiac ROS production, lead to detriments in oxidative reserve, and enhanced susceptibility to hypoxic challenge – all of which can be reversed using mitochondria targeted

antioxidants. The results presented here extend previous research (focused specifically on cardiac remodeling) by demonstrating metabolic and mitochondrial alterations in this unique form of heart disease. Maintenance of mitochondrial oxidative function is key in tailoring therapeutic approaches to limit cardiotoxic effects in treating the primary malignancy as well as limiting tumor-specific cardiomyopathy (Figure 7B). This is paramount in a clinical setting because cancer patients have increased occurrence of both myocardial ischemia[79] and adverse cardiac events[80] compared to age-matched population norms. We now provide evidence for the utility of mitochondrial antioxidants to combat cancer-induced oxidative impairments in the myocardium. Others have attempted to use pharmaceutical approaches to treat excessive ROS production in heart disease[81–83] but present with adverse effects on physiological ROS signaling in multiple other systems[84]. Our results suggest two pathways involved in pathological accumulation of free radicals through 1) loss of functional expression of mt-mRNA translation products for OXPHOS and 2) downregulation of GPx proteins for H_2O_2 clearance. An alternative approach to combat excess ROS may be utilizing alternative pharmaceuticals which could indirectly reduce myocardial ROS[85].

Conclusion

In summary, we present evidence of mitochondrial alterations contributing to ROS generation during cancer-induced cardiac atrophy *in-vivo*. We build on these results by demonstrating factors produced by the tumor cells contribute to reduction in cardiomyocyte oxidative reserve *in-vitro*. With heart disease and cancer competing for the leading cause of mortality worldwide, the contribution of malignancy to cardiomyopathy must be made clear and approaches to mitigate it researched. Further research should focus on the compounding effects of cancer and

chemotherapeutics on cardiac function and how other cancers less associated with cachexia may still contribute to heart disease.

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Table 1. Descriptive statistics of heart weight.

	Heart wet weight (mg)
PBS	120.08±3.15
LLC	108.21±3.36*

Data represent M±SEM. * - $p < 0.05$ compared to PBS.

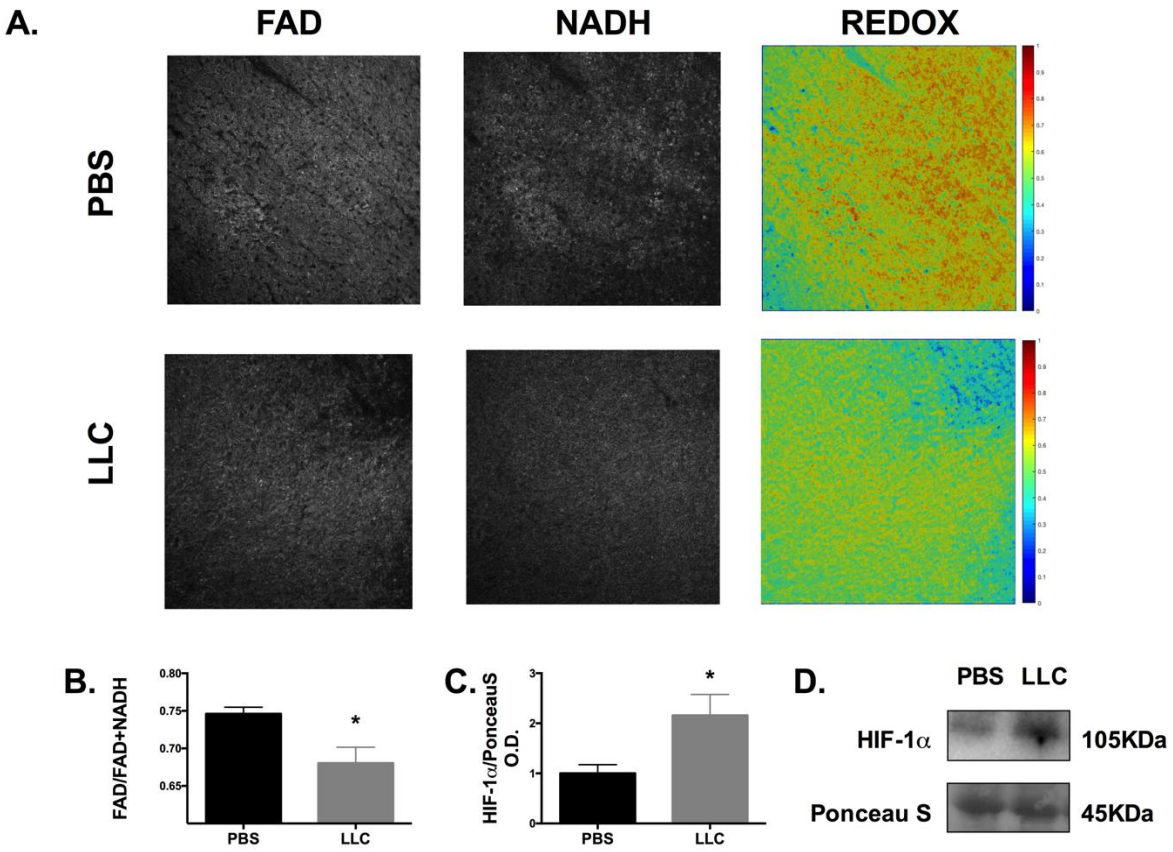

Figure 1. Cancer-induced cardiac atrophy demonstrates metabolic characteristics of heart disease. (A) Representative images of FAD, NADH, and redox ratio of TPEF of endogenous redox species within the myocardium of PBS and LLC left ventricle sections. (B) Calculated optical redox ratio and (C) HIF-1α protein content of PBS and LLC hearts. * indicates $p < 0.05$.

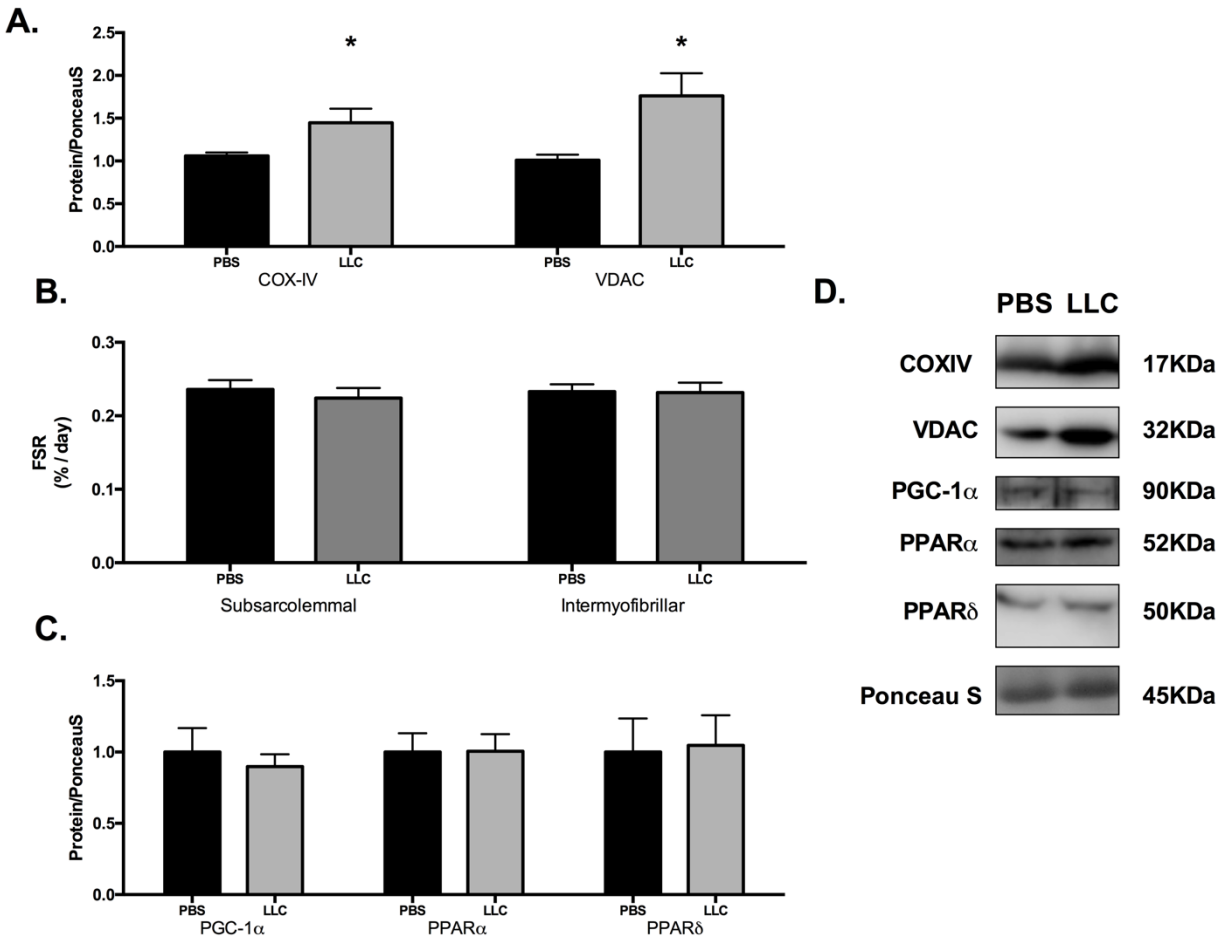

Figure 2. Cancer-induced cardiac atrophy alters mitochondrial content markers but not mitochondrial biogenesis protein content. (A) Markers of mitochondrial content, (B) subsarcolemmal and intermyofibrillar mitochondral FSRs and (C) markers of mitochondrial biogenesis in PBS and LLC hearts. (D) Sample immunoblot images of protein target indicated. Bands were cropped at indicated molecular weight and to show each group side-by-side. * indicates $p < 0.05$.

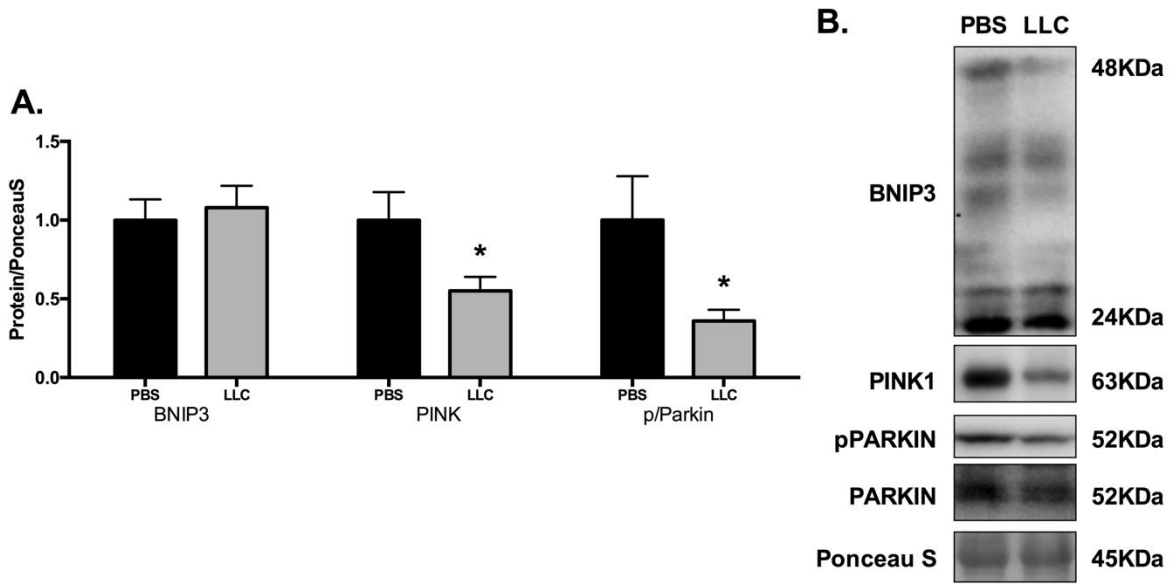

Figure 3. (A) Cancer-induced cardiac atrophy disrupts mitochondrial clearance. Markers of mitochondrial autophagy BNIP3, PINK, and phosphorylated to total Parkin in PBS and LLC hearts. (B) Sample immunoblot images of protein target indicated. Bands were cropped at indicated molecular weight and to show each group side-by-side. * indicates $p < 0.05$.

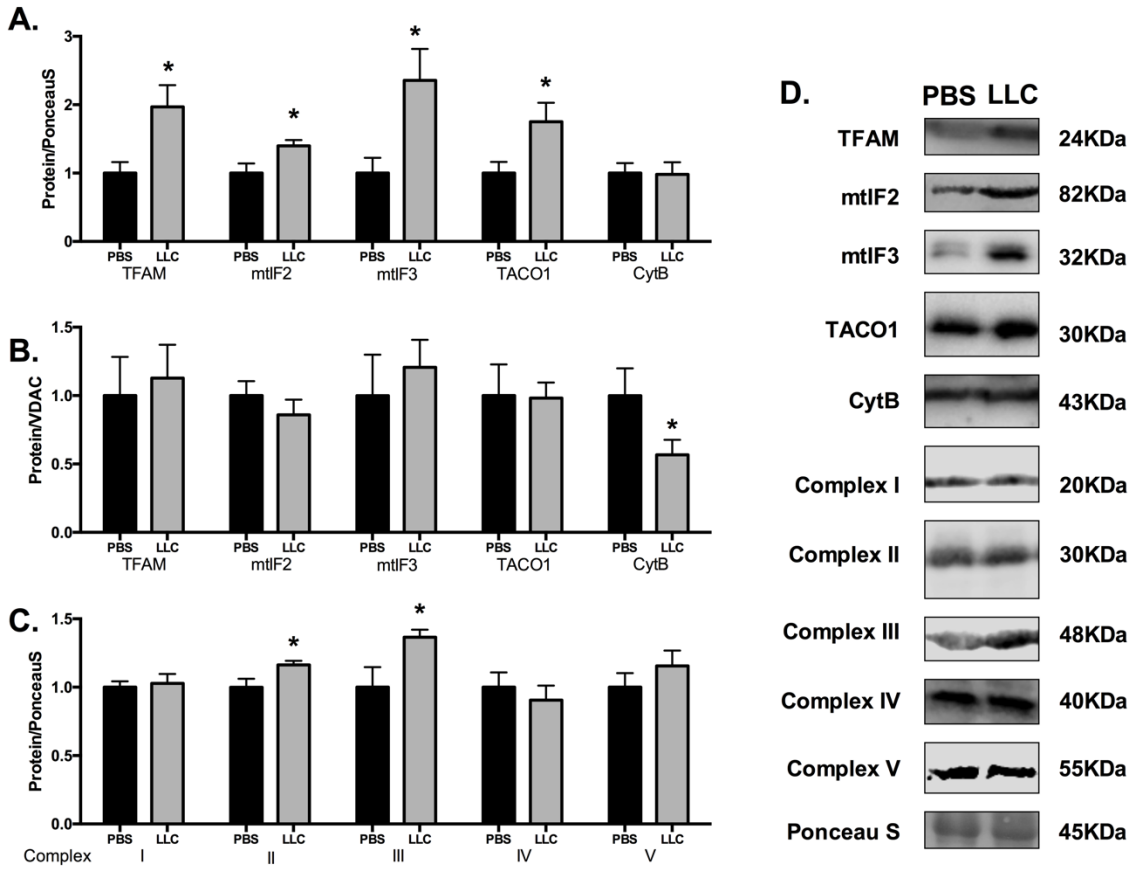

Figure 4. Cancer-induced cardiac atrophy disrupts mt-mRNA translation products but not mt-mRNA translation machinery. (A) Mitochondrial transcription and translation machinery (TFAM, mtIF2, mtIF3, TACO1) and a mt-mRNA translation product (CytB) normalized to total protein content by PonceauS and (B) normalized to mitochondrial content marker COX-IV in PBS and LLC hearts. (C) Immunoblot analysis of OXPHOS complex content as determined through protein subunits required for complex formation. Specific target for each subunit is indicated in the materials and methods section. (D) Sample immunoblot images of protein target indicated. Bands were cropped at indicated molecular weight and to show each group side-by-side. * indicates $p < 0.05$.

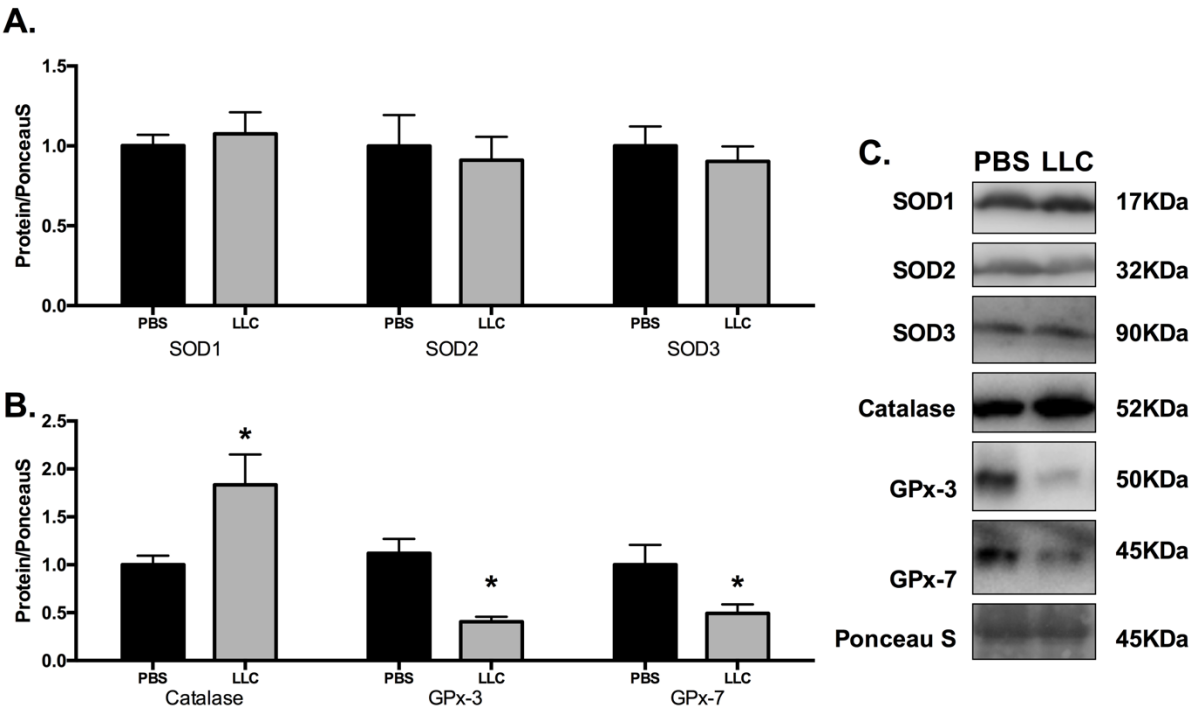

Figure 5. Cancer-induced cardiac atrophy disrupts hydrogen peroxide clearance protein content. (A) Superoxide dismutase protein isoforms and (B) hydrogen peroxide mitigation proteins catalase, and glutathione peroxidases -3 and -7 in PBS and LLC hearts. (C) Sample immunoblot images of protein target indicated. Bands were cropped at indicated molecular weight and to show each group side-by-side. * indicates $p < 0.05$

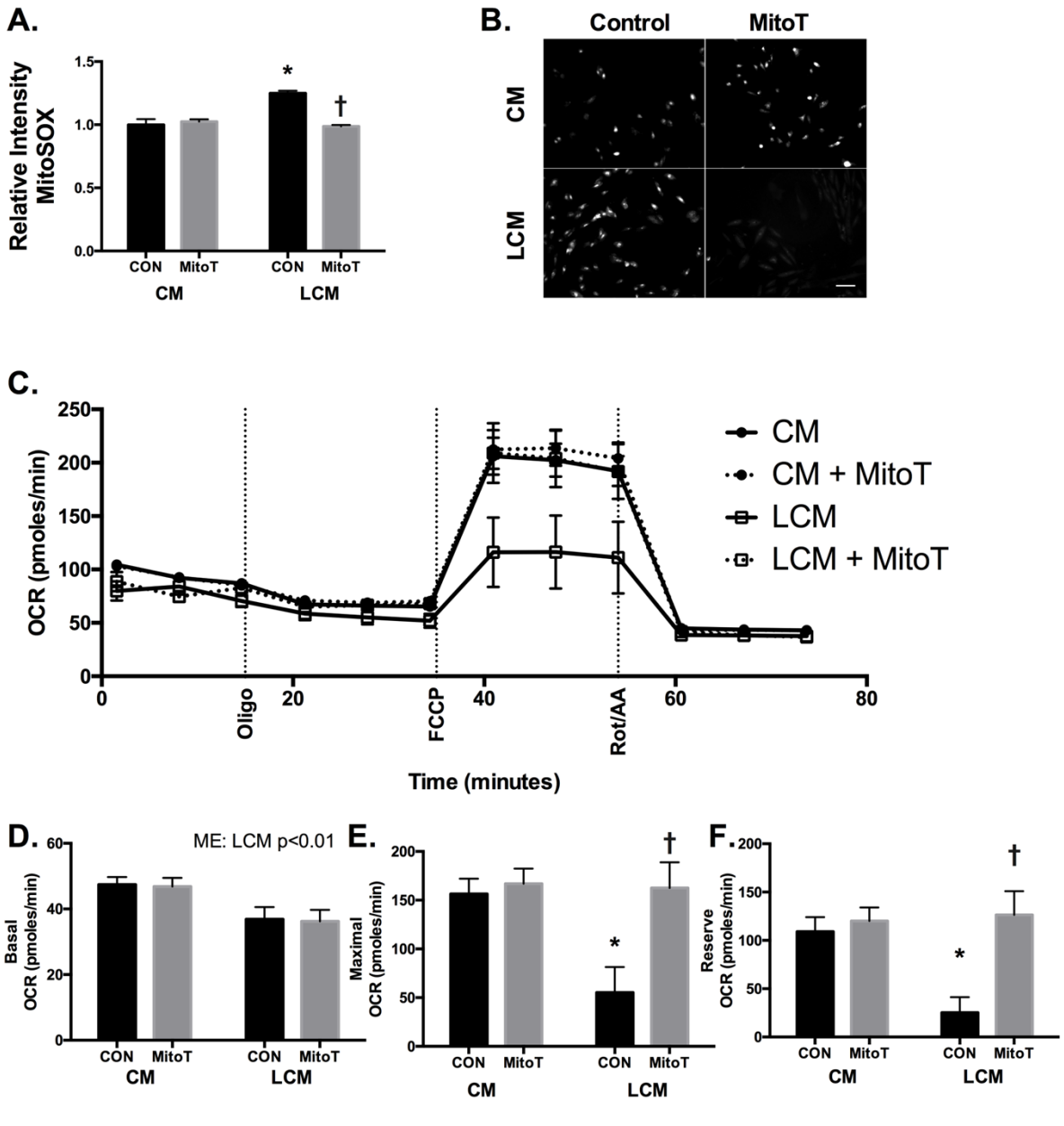

Figure 6. Media from LLC cancer cells contribute to greater ROS and reduced oxidative capacity. H9c2 cells were treated with a combination of LLC media (LCM) and 2μM of the mitochondrial targeted antioxidant (MitoT) for 2 hours and ROS assessed using MitoSOX (A,B) or similar treatment for 24 hours analyzing cellular bioenergetic flux analysis (C) to analyze oxygen consumption rates at (D) basal and (E) maximal rates, or calculated reserve rates (F). Scale bar = 100μm. * indicated $p < 0.05$ vs. control media (CM) with no MitoT (con); † indicated $p < 0.05$ vs. con cells receiving same media condition.

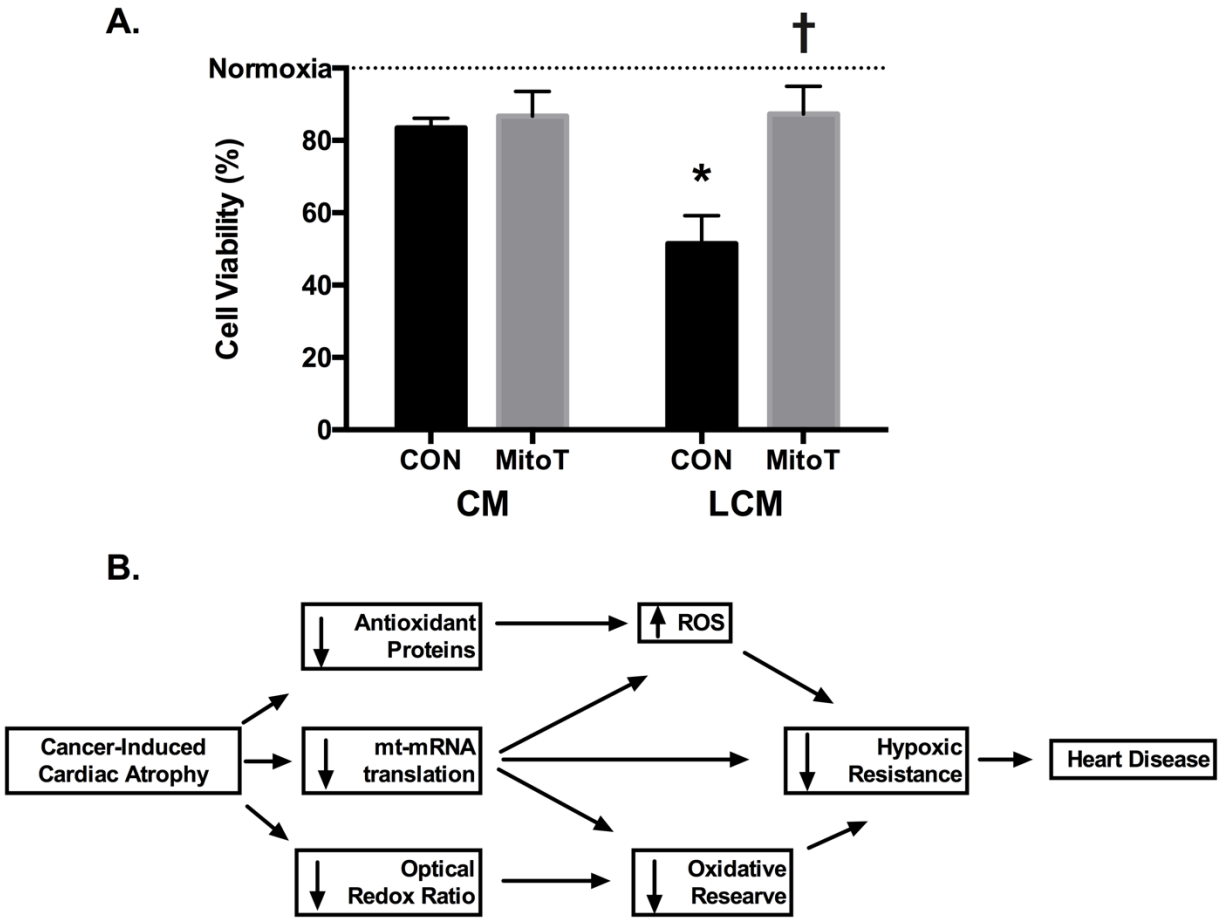

Figure 7. Media from LLC cancer cells reduces resistance to hypoxic insult in cardiomyocytes. (A) H9c2 cells were treated with a combination of LLC media (LCM) and 2μM of the mitochondrial targeted antioxidant (MitoT) for 24 hours and exposed to 1% oxygen and serum-free media for 6 hours followed by MTT viability assessment. * indicates $p < 0.05$ vs. control media (CM) with no MitoT (con); † indicates $p < 0.05$ vs. con cells receiving same media condition; dashed line at 100% indicates control cells that were maintained ~21% O_2 in standard growth media. (B) Graphical representation of suggested relationship between cancer-induced cardiac atrophy and oxidative characteristics.

Chapter 4 – Manuscript 2

Title: Mitochondrial Translation Initiation Factor 2 is Necessary for Cardiac Oxidative Capacity as Evident during Age-induced Cardiac Hypertrophy

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Running Title: mtIF2 and oxidative capacity in cardiac hypertrophy

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Abstract: Age and obesity each contributes to cardiac hypertrophy in a unique manner as it pertains to mitochondrial electron transport. Electron transport complexes I and IV are implicated in defective electron transport during cardiomyopathy and contain the majority of protein subunits that are transcribed and translated within the mitochondria. **Purpose:** To assess myocardial mt-mRNA translation factors in relation to mitochondrial content and mtDNA-encoded protein products using a mouse model of aged obesity and to test the necessity of mt-mRNA translation initiation factor 2 (mtIF2) in maintaining oxidative capacity and the cellular oxidation-reduction (redox) state in cardiomyocytes. **Methods**: C56BL/6J mice fed lean or high fat diet were aged to either ~3 months or ~22 months and the myocardium assessed using immunoblot and qPCR to determine differences in mitochondrial mRNA translation machinery. Using H9c2 cardiomyocytes, mtIF2 was knocked-down and two-photon excitation fluorescence (TPEF) of optical redox ratio (FAD/NADH + FAD), oxygen consumption, and hypoxic resistance was tested. **Results**: Aged mouse hearts were larger and contained less mtIF2 protein alongside reduced content of proteins encoded by mtDNA (CytB). Reducing the content of mtIF2 is associated with reduced oxidative characteristics such as OXPHOS complex I and IV content, optical redox ratio, oxygen consumption, and viability following hypoxia. **Conclusion:** We present evidence of altered mt-mRNA translation during cardiac hypertrophy in aged obesity. We build on these results by demonstrating the necessity of mtIF2 in maintaining oxidative characteristics of cardiac muscle cells.

Keywords: Cardiac Hypertrophy; Optical redox Imaging; bioenergetics; Hypoxia-reoxygenation; mitochondrial quality.

Introduction

Aging and obesity -- by themselves -- both contribute to cardiovascular diseases (CVD) such as congestive heart failure and coronary artery disease (8, 25); however, when combined in aged, obese patients, the risk for cardiac event, CVD mortality and all-cause mortality is greater than either condition alone (4, 35, 49). Each of these conditions are particularly relevant because of the increasing prevalence of both aging and obesity in recent years (5, 33). Being both advanced in age and obese each contributes to cardiac hypertrophy in a unique manner (6). Counterintuitively, epidemiological data suggests that obese patients with diagnosed heart disease have improved mortality rates compared to their age-matched, lean counterparts (1). This 'obesity paradox' is thought to be a result of greater circulating plasma free fatty acids contributing to the pool of metabolic substrates in the myocardium. This reduces the contribution of glycolysis to total ATP production and prevents the shift towards a more glycolytic phenotype that is characteristic of heart failure (11, 15, 45). This suggests a unique set of metabolic stressors that is placed on the myocardium of aged, obese cardiac patients.

Heart failure is characterized by a reduction in mitochondrial oxidative capacity and an associated increase in mitochondrial production of reactive oxygen species (ROS) due to inefficient electron transport through oxidative phosphorylation complexes (OXPHOS) (47). Deleterious OXPHOS byproducts produced over the lifespan may contribute to mutations in mitochondrial DNA (mtDNA) which is particularly susceptible due to its close proximity to electron transfer, lack of dedicated repair machinery, and propagation of mutated mtDNA copies (21, 24, 37). However, some research suggests mtDNA mutations may not result in greater deleterious OXPHOS byproduct production nor lead to the aging phenotype (39, 50). It is, thus,

unclear if greater ROS is a consequence of another process that alters electron transport efficiency in the diseased myocardium.

Reductions in cardiac oxidative capacity have been explained, in part, by reduced electron transport through complexes I and IV of the electron transport chain (ETC) (34) which contain a majority of the subunits encoded by mtDNA (10). Alterations in mtDNA-encoded subunit expression can alter the balance between nuclear and mitochondrial constituents of the ETC and generate non-functional complexes (48). The process of mitochondrial-mRNA (mt-mRNA) translation is responsible for synthesizing and inserting the 13 protein subunits of the ETC that remain encoded by mtDNA (22, 42). mt-mRNA translation is governed by a discrete set of translation machinery within the mitochondrial matrix including mitoribosomes, mt-tRNAs, and a specialized set of nuclear-encoded proteins to orchestrate the process (31, 32). These proteins include mitochondrial initiation factors (mtIFs), elongation factors (mtEFs), and translation activators that help govern each step of the mt-mRNA translation process and is directly tied to ETC capacity and thus production of ROS in the myocardium.

Mutations in key controllers of mt-mRNA translation result in altered ETC capacity thereby leading to greater production of ROS (42). Furthermore, we have demonstrated that gene and protein expressions of mt-mRNA translation machinery are subject to change from metabolic perturbations in various tissues (20, 28, 40) suggesting that mt-mRNA translation may be dysregulated during cardiomyopathy. Previous research establishes a link between elevated levels of ROS production and mtDNA mutations (37, 46) leading to early onset heart failure (51), and decreases in lifespan (18, 30). One approach to mitigate oxidative stress of heart disease may be to control the mt-mRNA translation/ETC/ROS production circuit (8); however,

no prior connections between mt-mRNA translation machinery and oxidative capacity have been determined.

In an effort to better understand how mt-mRNA translation machinery affects oxidative characteristics of the myocardium, the purpose of this investigation was to assess myocardial mt-mRNA translation factors in relation to mitochondrial content and mtDNA-encoded protein products using a mouse model of aged obesity. By using an established, *in vivo* model of cardiac hypertrophy, we were able to determine alterations attributable to aging, high fat diet-induced obesity, and a combination of both conditions. Additionally, we used murine cardiomyocytes *in vitro* to test the necessity of mt-mRNA translation initiation factor 2 (mtIF2) in maintaining oxidative capacity and the cellular oxidation-reduction (redox) state in cardiomyocytes. We demonstrate significant alterations in mt-mRNA translation associated with age- and obesity-induced cardiac hypertrophy and use a variety of approaches to show mtIF2 is required to maintain functional characteristics of cardiomyocytes. This investigation provides novel insight into the connection of mt-mRNA translation to ETC components for control of cardiac oxidative capacity and has implications for ROS production, mtDNA mutation rates and aging.

Materials and Methods

Animal model of aged, obese mice

All methods were approved by the Institutional Animal Care and Use Committee at the University of Arkansas. Thirty-two, male C57BL/6J mice were a generous gift from Rigel Pharmaceuticals and were housed in the University of Arkansas Central Laboratory Animal Facility. Animals were kept on a 12:12-hour light-dark cycle and given access to either normal (NC, 17% fat, Teklad 22/5 Rodent Diet, 86140, Teklad Diets, Madison, WI)) or high-fat (HF, 60% kcals fat, D12492, Research Diets, Inc., New Brunswick, NJ) chow beginning at 3 weeks of

age. The study consisted of four groups: young lean (YL), young obese (YO), aged lean (AL), and aged obese (AO) (n = 8 per group). Mice that were 3-4 months and 22-24 months old were considered young and aged, respectively. Mice were given access to water and chow *ad libitum*. Following euthanasia, heart samples were snap frozen and stored at -80^{o}C before further analysis.

Immunoblot analysis

Protein was extracted from small fractions of muscle taken from the hearts, separated by 8% or 12% SDS-PAGE and transferred to polyvinylidene fluoride membrane as described (9, 19) alongside molecular weight ladder (BioRad #1610394). Membranes were blocked using 5% milk in TBS (w/v) with 0.2% tween and incubated with specific primary antibodies at 4°C overnight. Primary antibodies were specific to COX-IV (Cell Signaling Technologies, #4844), PGC-1α (Santa Cruz Biotechnologies, sc-13067), TFAM (Cell Signaling #7495), mtIF2 (Santa Cruz #365477), mtIF3 (Origene TA800421), mtEF-Tu (TUFM, Abcam ab67991), TACO1 (FLJ36733, Abcam ab 121688), Cyt-B (Santa Cruz 11436), ND4 (Santa Cruz #20499), VDAC (Cell Signaling #4866), AMPK (Cell Signaling #2793), phosphorylated $AMPK_{Thr172}$ (Cell Signaling #2535), Total OXPHOS Cocktail (Abcam ab110413). Using appropriate HRP-conjugated or fluorescent conjugated secondary antibodies, protein antigens were visualized within a linear range by either ECL on Protein Simple FluorChemM (Minneapolis, MN) or near-infrared fluorescence on Odyssey Fc (LI-COR, Lincoln, NE) and analyzed using ImageStudio Software (LI-COR). Bands were normalized to the 45kDa Actin band of PonceauS as loading control. For each experiment, all groups were represented on each membrane and normalized to control.

Gene Expression Analysis

RNA isolation followed by reverse transcription real time polymerase chain reaction was performed as previously described (20, 27, 29). Briefly, RNA was isolated using Trizol ragent and Ambion RNA Isolation Kit (Ambion, ThermoFisher Scientific, Grand Island, NY, USA), reverse transcription was performed using 1µg RNA and Superscript VILO cDNA synthesis kit (11754, Invitrogen) according to manufacturer protocol. cDNA was amplified in 25µL reaction containing TaqMan Gene Expression Mastermix and appropriate fluorescent probe for *18s* (Mm03928990), or using SYBR Green chemistry with previously described primer sets for 12S rRNA and 16S rRNA(28). Samples were incubated at 95°C for 4 min, followed by 40 cycles of denaturation, annealing, and extension at 95, 60, and 72°C. Fluorescence was measured at the end of the extension step for each cycle. Cycle Threshold (Ct) was determined and the ΔCt value calculated as the difference between Ct value and 18s Ct value. 18S Ct was not different among experimental groups. Final quantification of gene expression was calculated using the ΔΔCt method. Relative quantification was calculated as $2^{\Delta\Delta Ct}$.

H9c2 Culture experiments

H9c2 ventricular cardiomyocytes were purchased from ATCC (CRL-1446) and grown at 37^0C, 5%CO_2, and 20% O_2 in DMEM containing 10% FBS and 1% pen/strep and changed every second day. When cells were ~75% confluent, $5x10^4$ cells/well were sub-cultured in 6-well plates for 24 hours before liposome-mediated gene transfer was performed as previously described (29). Briefly, 1µg of shRNA-mtIF2 (sh-mtIF2; Catalog #RSH045068, Genecopeia, Rockville, MD, USA) or a shRNA scramble control sequence (sh-con) was diluted in 50µL reduced serum media combined with Lipofectamine 2000 (ThemoFisher Scientific). Cells were incubated for 5 hours before rinsing and replacement with standard growth media.

Two-photon excitation fluorescence of endogenous NADH and FAD

Two-photon excitation fluorescence (TPEF) was used to measure endogenous fluorescence of NADH and FAD as previously described (2). Briefly, laser excitation by MaiTai Ti:Sapphire laser source (Spectra-Physics, Santa Clara CA) was tuned to 755nm or 860nm. Images were acquired using a resonant-galvo scanner and GaAsP photomultipler tubes (H7422-40, Hamamatsu) with 460/40 nm(NADH), 525/45 nm(FAD), and 600/70 nm (Rhodamine) filters. 10μm section of the left ventricle were cut along the short axis. Slides were imaged (512x512 pixels, 16-bit depth, 260 μm^2) and the pixel-wise FAD/(NADH+FAD) ratio normalized to rhodamine was calculated. Multiple fields were imaged for each sample and averaged to represent an individual biological sample. NADH and FAD image analysis was completed using MATLAB (MathWorks, Natick, MA).

Bioenergetic flux analysis

Oxygen consumption rates (OCR) were analyzed using Seahorse XFp extracellular flux analyzer (Agilent, Santa Clara, CA) according to manufacturer instructions and as previously described (3). Briefly, 24 hours following shRNA transfection, cells were trypsinized, rinsed with PBS, and $2x10^4$ cells were seeded per well in growth medium. After 24 hours incubation, media was removed and replaced with Seahorse Assay media containing 7 mM glucose, 2 mM pyruvate, and 2 mM glutamine. OCR was measured prior to and following sequential addition of 1 μM oligomycin, 1 μM FCCP, and 1 μM rotenone/Antimycin A. This allowed assessment of cellular OCR related to basal respiration and maximal uncoupled respiration. Reserve respiration was determined as the difference between maximal and basal after normalizing to non-mitochondrial oxygen consumption.

Hypoxic exposure and MTT viability assessment

To assess the resistance of H9c2 cells to hypoxia similar to that seen during ischemic challenge, 48 hours following shRNA transfection, media was replaced with serum-free DMEM and cells were placed in a dual gas controlled (Oxycycler C42, Biospherix, Parish, NY) incubator sub-chamber. Oxygen was flushed by nitrogen and maintained at 1% O_2 and 5% CO_2 for 6 hours. The combination of serum-free media with a hypoxic environment was used to simulate ischemic conditions (26). Following hypoxic exposure, 1 mM MTT (M6494, Invitrogen) was added to cells and incubated ~20% O_2 for 2 hours. The resulting formazan crystals were solubilized by addition of 1000 μL of 350 mM SDS in 0.01% HCl and absorbance was read at 570 nm. A separate plate was maintained at ~20% O_2 to serve as 100% viability control.

Statistical Analysis

Statistics were calculated and visualized using GraphPad Prism v6.0. For animal studies, a 2x2 analysis of variance was used to compare conditions of age (young vs. aged) by diet (lean vs. obese). Where significant interactions occurred, Fisher's LSD post hoc analysis was used to determine differences between groups. To compare shRNA-control to shRNA-mtIF2, Student's t-test was used. For all experiments, α was set at 0.05 and data presented are mean ± SEM.

Results

The obesity, aging, and cardiac hypertrophy phenotype

Phenotypic data regarding animal age, body weight, tibia size, and heart weights from this animal cohort have been previously published. Overall, the high-fat diet animals were significantly heavier than their age-matched lean counterparts. Absolute and relative to tibia

length, heart weight showed a main effect of age (~20% greater) and diet (~20% greater) as being significant contributors to elevated heart mass.

Mitochondrial content alterations in the myocardium during aging and obesity

To assess mitochondrial content of the heart in this animal model, we used immunoblot analysis of the surrogate marker COX-IV as well as controllers of mitochondrial biogenesis and replication, PGC-1α and TFAM. COX-IV protein was ~40% greater in both aged groups compared to young ($p < 0.05$) with no significant effect of diet ($p > 0.05$; Figure 1A). PGC-1α protein content was differentially expressed by both high-fat diet and age. YO and AL groups showed ~2-fold and 1.5-fold greater content of PGC-1α compared to YL and AO groups ($p < 0.05$) while there was no difference between YL and AO groups ($p > 0.05$, Figure 1B). No significant differences were found in the protein content of TFAM between any groups ($p < 0.05$, Figure 1C).

Mitochondrial mRNA-translation alterations in the myocardium during aging and obesity

To understand the effects of aging and obesity on the process of mt-mRNA translation in the myocardium, we analyzed key controllers of the mt-mRNA translation process. First, we examined major components of the mitochondrial ribosomal subunits, mitochondrial 12S rRNA content was ~30% lower in high fat diet groups compared to lean ($p < 0.05$) and ~45% lower in aged groups compared to young ($p < 0.05$, Figure 2A). In the YO group, the 16S rRNA was only ~25% that of the YL control ($p < 0.05$). Both aged groups had ~30% lower 16S rRNA compared to the YL controls ($p < 0.05$) but were not different than each other ($p > 0.05$, Figure 2B). High fat diet (~25%) and aging (~40%) both showed significantly lower levels of mtIF2 protein compared to lean and young controls, respectively ($p < 0.05$, Figure 2C). mtIF3 protein content was ~50% greater in AO group compared to YO and AL ($p < 0.05$) but when compared to YL,

this did not reach statistical significance ($p = 0.083$, Figure 2D). mtEF-Tu protein content was not different between young groups but the AL group was about 2-fold greater when compared to AO ($p < 0.05$, Figure 2E). TACO1 content was ~35% lower in both high fat diet groups compared to their lean counterparts, with no significant effect of age ($p < 0.05$, Figure 2F). Mitochondria-encoded CytB protein levels were ~25% lower in high fat diet groups compared to lean controls and ~25% lower in aged groups compared to young controls ($p < 0.05$, Figure 2G). When normalized to protein levels of COX-IV to account for mitochondrial content, CytB was ~45% lower in both aged groups compared to young counterparts ($p < 0.05$, Figure 2H).

Genetic downregulation of mtIF2 alters oxidative capacity of cardiomyocytes

In order to assess the necessity of mtIF2, and thus limitations in mitochondrial mRNA translation, on oxidative capacity of cardiac muscle cells, we utilized a transient knockdown approach *in vitro* using H9c2 cardiomyocytes. Immunoblot analysis of sh-mtIF2 transfected cells reveals 22% lower protein content of mtIF2 compared to sh-Con ($p < 0.05$; Figure 3A). COX-IV and VDAC were used as markers of mitochondrial content with no differences found between sh-Con and sh-mtIF2 cells ($p > 0.05$; Figure 3A). The ratio of phosphorylated AMPK to total AMPK was significantly elevated by >2-fold in sh-mtIF2 cells compared to sh-Con ($p < 0.05$; Figure 3A). Cyt-B and ND4 are two proteins translated by mt-mRNA translation machinery and were found to be significantly lower in sh-mtIF2 compared to sh-Con by ~25% and ~15%, respectively ($p < 0.05$; Figure 3A). OXPHOS complex assembly was assessed by analyzing content of core proteins within each complex that are actively degraded when the complex is not adequately assembled. Markers for complexes I and IV had ~15-20% lower protein content in sh-mtIF2 compared to sh-Con ($p < 0.05$) with no differences seen between groups for markers of complex II, III, or V ($p > 0.05$; Figure 3B). TPEF was used to visualize the optical

oxidation/reduction state of the H9c2 myocytes. The ratio of FAD/(FAD + NADH) was significantly lower in sh-mtIF2 by ~30% ($p < 0.05$, Figure 4 A, B). Bioenergetic analysis revealed lower levels of basal OCR (~50%), maximal OCR (~30%), and reserve capacity (~40%) in sh-mtIF2 cells compared to sh-Con ($p < 0.05$, Figure 4 C, D). Upon exposure to 1% O_2 for 6 hours, sh-mtIF2 cell viability was significantly lower (~20%) than that of control H9c2 cells ($p < 0.05$; Figure 5).

Discussion

This investigation highlights specific alterations in mt-mRNA translation machinery during the pathogenesis of aging-induced and obesity-induced cardiac hypertrophy. Using a mouse model of aged obesity that has been previously characterized, we identified a loss of mtIF2 despite markers of greater mitochondrial content (COX-IV; Figure 2) alongside reductions in Cyt-B, a protein encoded by the mtDNA, suggesting impaired mt-mRNA translation in aged obesity. Furthermore, using *in vitro* genetic manipulation of cardiomyocytes, we identified the necessity of mtIF2 in maintaining oxidative characteristics of cardiac muscle (Figures 3-5). These results extend previous research on the metabolic alterations associated with aging-induced mitochondrial irregularities (13, 14, 41, 45). Specifically, novel evidence is presented on the contribution of mt-mRNA translation machinery to maintaining oxidative capacity in the myocardium. These data demonstrate a clear contribution of mtIF2 to the unique cardiomyopathy seen in aged obesity with implications on oxidative capacity, ROS production, and mtDNA mutations – all of which have been connected to the decline in cardiac function over time.

Implications for mtIF2 during Cardiomyopathy

In maintaining 13 ETC-subunit encoding genes in mtDNA, dedicated machinery to transcribe and translate each gene into a protein becomes necessary. We have assessed this machinery by analyzing regulators of mt-mRNA translation ribosomes, initiation, elongation, and the only known mammalian translational coactivator. From these analyses, we identified detriments in mtIF2 and mt-rRNAs during a form of cardiomyopathy. Bacterial and eukaryotic, cytoplasmic mRNA translation each contain three translation initiation factors; however, mammalian mitochondria contain only two with no currently known equivalent of the initiation factor 1 (IF-1) (54). Functionally, the mammalian mtIF2 appears capable of replacing both IF-1 and -2 of *E. Coli* resulting from a 37 amino acid insertion from IF-1 into mammalian mtIF2 allowing mtIF2 to bind the start codon (fMet-tRNA), GDP, and the small ribosomal subunit to generate a pre-initiation complex (16). This predicates mtIF2 as a primary controller of mt-mRNA translation initiation. Heart disease has been associated with metabolic changes in the myocardium including a decrease in electron transport through ETC complexes I and IV (34) – two complexes that include >75% of the mtDNA-encoded proteins (32). Alterations in the expression of mtDNA-encoded subunits through changes in mt-mRNA translation are one potential mechanism contributing to metabolic irregularities present in cardiomyopathies. In support of this, we present evidence that mtIF2 protein and the mitochondrial rRNAs are downregulated in the myocardium by both aging and obesity and that this is concomitant with similar changes in CytB protein levels (a protein encoded by mtDNA) despite greater markers of mitochondrial content in aged hearts (Figures 1, 2). These results suggest detriments in oxidative capacity and ETC components of the myocardium may directly result from impaired mt-mRNA translation initiation and reductions in necessary mt-mRNA translation machinery due to insufficient levels

of mtIF2 and mitochondrial rRNAs, respectively. Reductions in proper mt-mRNA translation would likely trigger deficient oxidative capacity and perhaps lead to greater ROS production, mtDNA mutation rate and mitonuclear protein imbalance leading to the mitochondrial unfolded protein response further propagating the ETC deficits (8, 23, 36). The culmination of these detriments, overtime, may contribute to the metabolic alterations associated with age-induced or obesity-induced cardiac hypertrophy.

Necessity of mtIF2 for maintaining cardiac oxidative capacity

Our *in vivo* data suggests a potential contribution of mtIF2 towards altered metabolic characteristics of the heart by limiting translation of mt-mRNA; therefore, we sought to more clearly define the connection of mtIF2 to specific cardiomyocyte functional outcomes. Using shRNA knockdown of mtIF2 in H9c2 cardiomyocytes, we present novel evidence for the necessity of proper protein expression of mitochondrial translation initiation machinery to maintain the oxidative characteristics of cardiac muscle. The optical redox ratio of FAD/(NADH + FAD) as visualized using TPEF is a non-destructive and label free method of live cell imaging that can ascertain relative contribution of glycolysis and oxidative phosphorylation (3, 38, 52). Using this approach, we are the first to report that reductions in protein content of mtIF2 can be directly associated with a relative increase in glycolytic utilization compared to oxidative phosphorylation (Figure 4). Furthermore, we demonstrate that insufficient expression of mtIF2 can reduce oxygen consumption capacity and exacerbate cardiomyocyte cell death following hypoxic exposure *in vitro* (Figure 4,5). These results have clear implications for pathological cardiac hypertrophy as seen during aging and obesity which both present with downregulated cardiac mtIF2 protein content (Figure 2).

mt-mRNA translation and Mitochondrial Quality Control

Traditional views of mitochondrial quality control encompass the processes of mitochondrial biogenesis, mitophagy, and mitochondrial dynamics (53). Recent published evidence has established other cellular processes and their importance for appropriate mitochondrial oxidative metabolism including the mitochondrial unfolded protein response (25), mitochondrial supercomplex formation (17), and mitochondrial mRNA translation (28, 44). Each of these cellular pathways contributes to the overall function and efficiency of the ETC in producing sufficient ATP without deleterious ROS production or a resulting increase in mtDNA mutation rate. Published literature predicates mitochondrial metabolism in combating the deleterious effects of aging on the heart (8) but these results, alongside other reports, suggest that maintenance of mt-mRNA translation machinery, specifically mtIF2, directly contributes to mitochondrial oxidative quality (7, 8, 48). Mitochondrial oxidative capacity should be a primary target to combat the deleterious effects of acquired cardiomyopathies including aged obesity-induced cardiac hypertrophy and this should be accomplished through the appropriate quality control mechanisms *including mt-mRNA translation.* Further research should aim to directly connect mitochondrial translational outcomes to the rates of mtDNA mutation and subsequent ROS production.

Conclusion

In summary, we present evidence of altered mt-mRNA translation during age-induced and high fat diet-induced cardiac hypertrophy. We build on these results by demonstrating the necessity of mtIF2 in maintaining the redox state and oxidative capacity of cardiac muscle cells. With aging and obesity each contributing to heart disease in a unique manner, the contribution of each towards metabolic cardiomyopathy must be made clear. Further research should focus on the

mitigating effects mtIF2 overexpression may have on cardiac mitochondrial function (oxidative capacity, ROS, mtDNA mutations) and how this may contribute to increased lifespan or reduced risk for cardiovascular event.

Conflicts of Interest: The authors declare no conflicts of interest associated with this publication.

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Author Contributions: DEL, TAW, NR and NPG conceived and designed research; DEL, JLB, RAP, MER, LAB, WSH, TAW, and NPG performed experiments; DEL, JLB, RAP, MER, NR, NPG analyzed data; DEL, JLB, RAP, NR and NPG interpreted results of experiments; DEL, and NPG prepared figures, drafted manuscript, edited and revised manuscript; All authors approved final version of manuscript.

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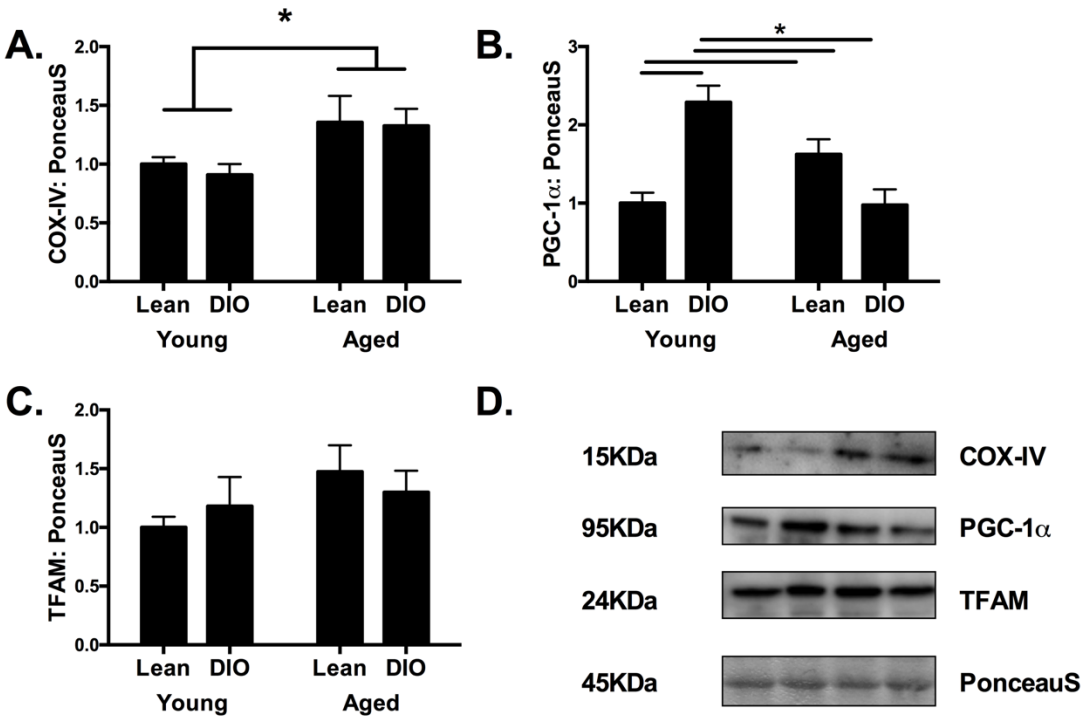

Figure 1. Effects of Aging and Obesity on Cardiac Mitochondrial Content. Immunoblot analysis of mitochondrial content marker COX-IV (A) and controllers of mitochondrial biogenesis (PGC-1α; B) and replication/transcription (TFAM, C). (D) Sample immunoblot images. Each group was represented equally across membranes and normalized across membranes using an internal control. * indicates $p < 0.05$ between groups connected by horizontal bar.

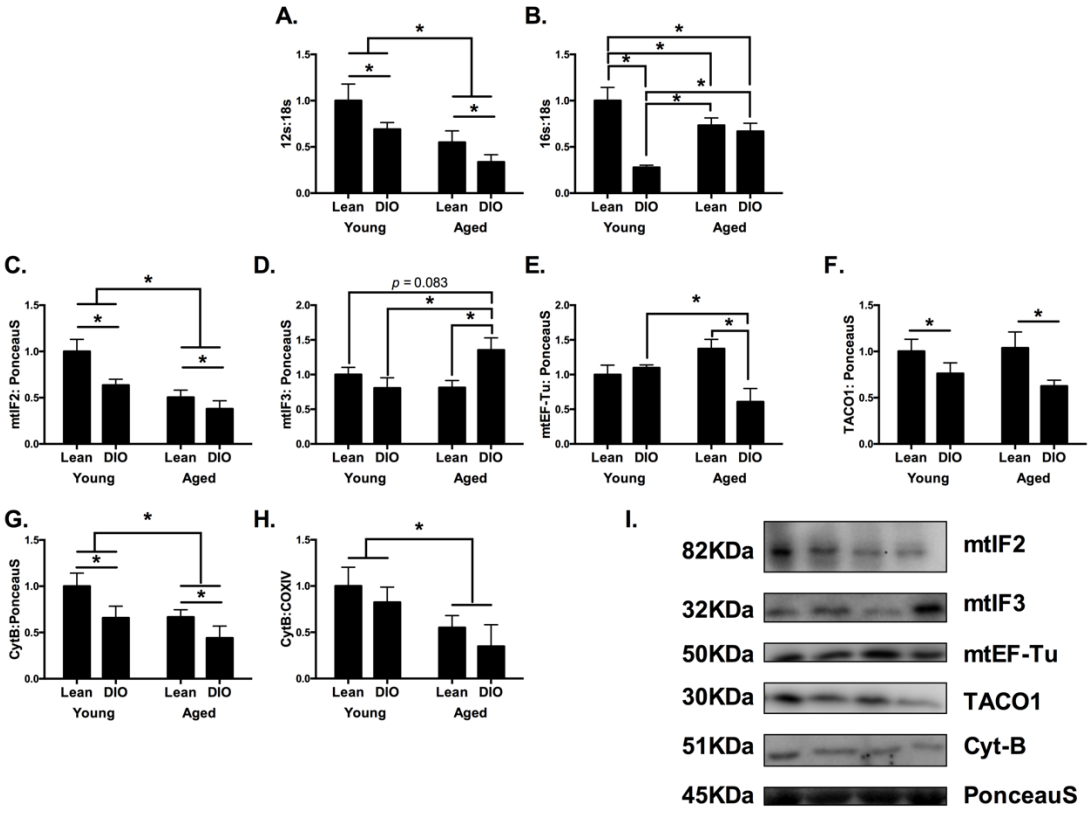

Figure 2. Effects of Aging and Obesity on Cardiac Mitochondrial mRNA translation. RT-qPCR analysis of mitochondrial rRNA content (A,B). (C-F) Immunoblot analysis of protein content of machinery involved in mitochondrial mRNA translation and CytB -- a protein encoded by mtDNA normalized to PonceauS (G) or mitochondrial content (H). (D) Sample immunoblot images. Each group was represented equally across membranes and normalized across membranes using an internal control. * indicates $p < 0.05$ between groups connected by horizontal bar.

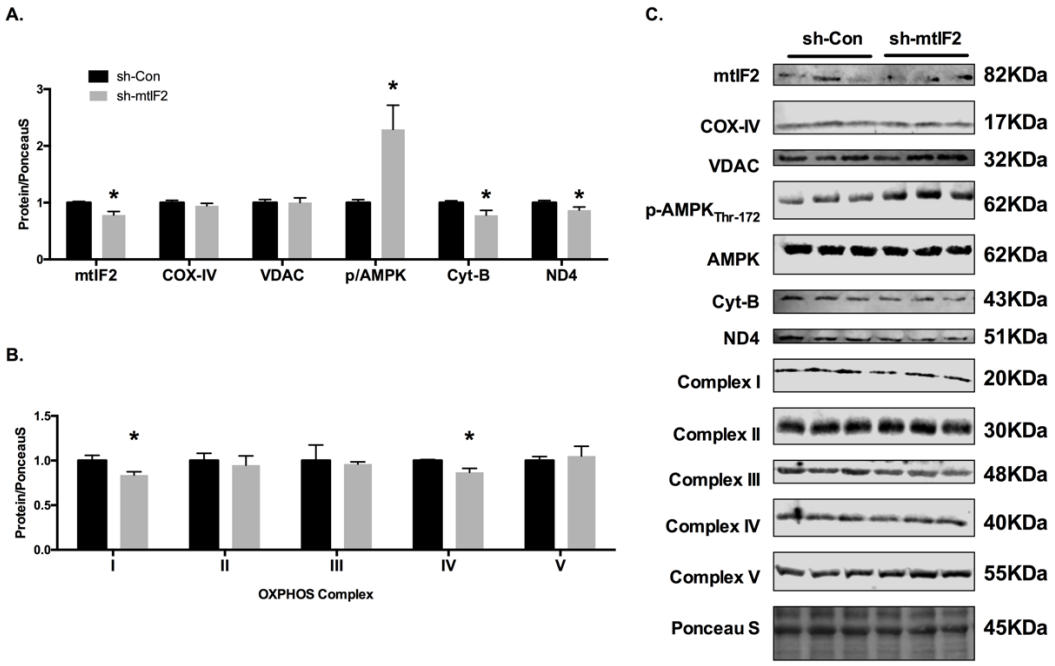

Figure 3. shRNA-mtIF2 alters protein content of markers of mitochondrial metabolism. (A) Immunoblot analysis of mtIF2, COX-IV, VDAC, phosphorylation ratio of AMPK, Cyt-B and ND4 in shRNA-Con and shRNA-mtIF2 transfected H9c2 cardiomyocytes and (B) mitochondrial OXPHOS complexes. (C) Sample immunoblot images. Analyses were performed in triplicate with multiple independent experiments. * indicates $p < 0.05$.

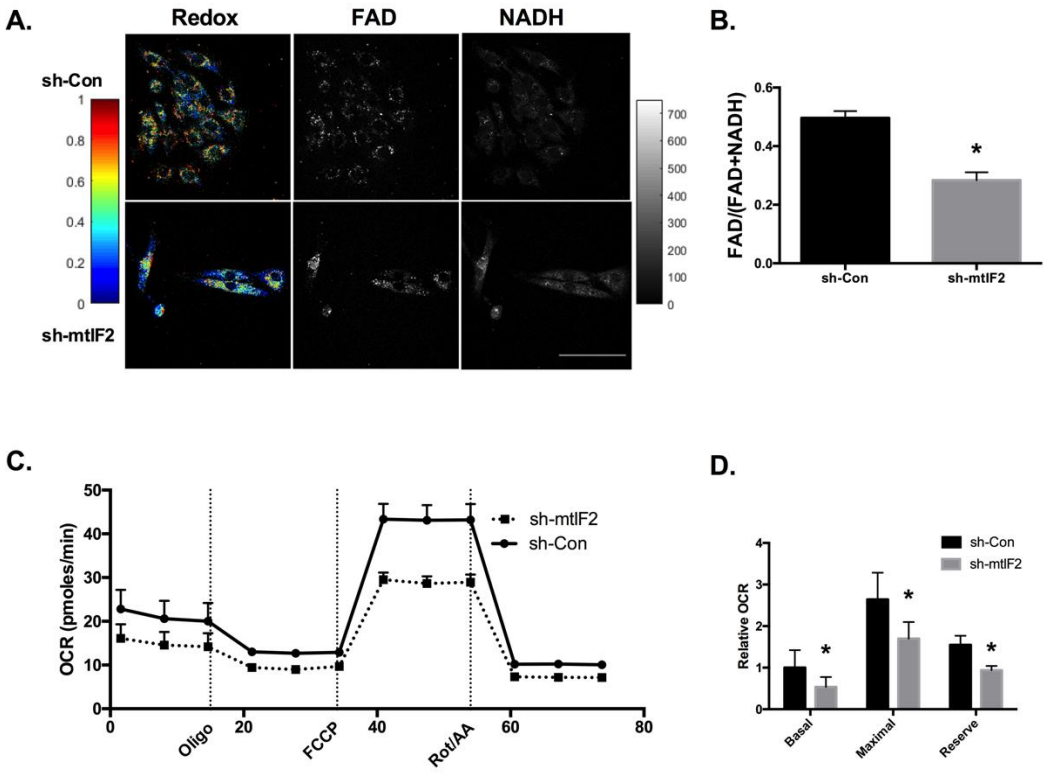

Figure 4. shRNA-mtIF2 alter oxidation/reduction state in H9c2 Cardiomyocytes. (A,B) TPEF of optical redox ratio [FAD/(FAD+NADH)] in H9c2 cardiomyocytes transfected with either shRNA-Control or shRNA-mtIF2. Bioenergetic flux analysis of H9c2 cells over time (C) and resulting relative OCR as a proportion of the control basal rate. Analyses were performed in triplicate with multiple independent experiments. * indicates $p < 0.05$.

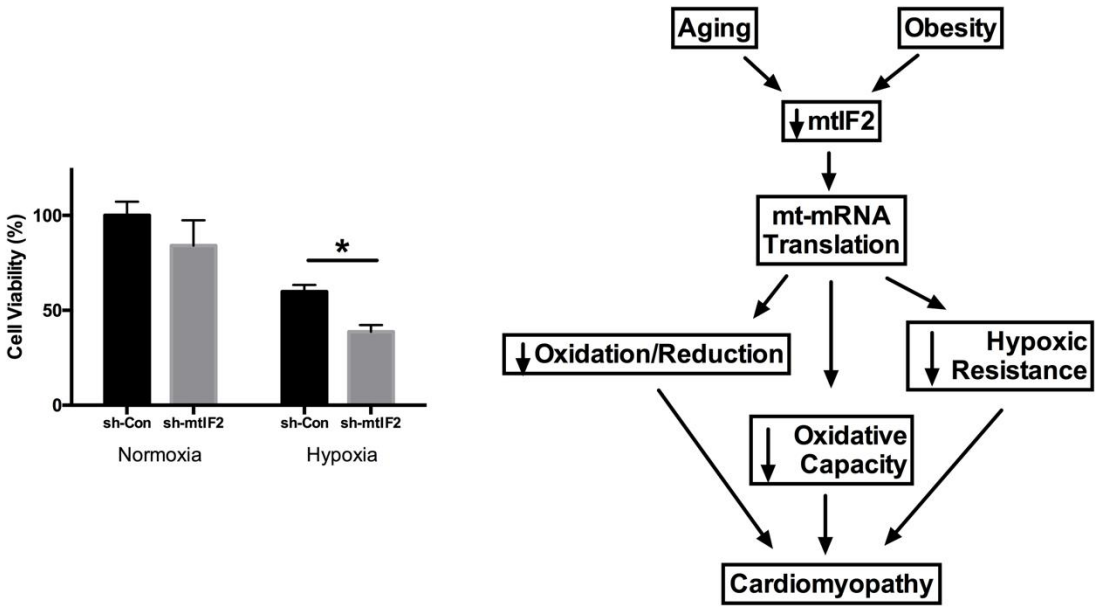

Figure 5. **(**A) mtIF2 contributes to cardiomyocyte hypoxic resistance. MTT assay of cell viability conducted after 6 hours of incubation at 1% O_2 in H9c2 cardiomyocytes transfected with either sh-Con or sh-mtIF2. Analyses were performed in triplicate with multiple independent experiments. * indicates $p < 0.05$. (B) Conceptual illustration of results.

Chapter 5 – Discussion

This series of experiments has established the importance of the mt-mRNA translation machinery in maintaining mitochondrial oxidative capacity in the myocardium. The specific aims outlined within this dissertation have determined 1) mt-mRNA translation is altered in an aging- and obesity-induced model of cardiac hypertrophy, 2) mt-mRNA translation is dysregulated in the unique cardiac atrophic state seen in advanced cancer and 3) mt-mRNA translation initiation through mtIF2 is required to maintain cardiac oxidative characteristics. We have accomplished these experiments through a combination of unique *in vivo* mouse models of heart disease followed by specific *in vitro* genetic approaches targeting mt-mRNA translation machinery using a variety of biochemical and functional assessments. The implications of these experiments extend areas of cardiometabolic disease, cardio-oncology, and the basic science of mitochondrial quality control mechanisms to maintain oxidative characteristics of the most mitochondria-dense tissue within the human body.

Until now, the primary focus of research into mitochondrial therapeutics to combat heart disease has been enhancing lipid utilization and improving mitochondrial quality control pathways (7, 22). The goal in targeting mitochondrial quality control pathways is to optimize the pool of mitochondria by eliminating non-functional organelles (mitophagy) (21) and optimizing the interconnectedness of the mitochondrial reticulum (dynamics) (4). Other attempts to promote mitochondrial biogenesis through overexpression of the master regulator PGC-1α have been limited because of a type of ceiling reached on mitochondrial content in the already extremely oxidative cardiac muscle (3, 11). These studies have suggested the insufficiency of approaches targeted towards canonical mitochondrial quality control mechanisms. The results of our investigations into mt-mRNA translation demonstrate the paramount importance of maintaining

the pool of mitochondrial OXPHOS subunits coded by mtDNA to maintain the integrity of the ETC. It should, therefore, be noted that mt-mRNA translation is now implicated as a key component of proper and sufficient mitochondrial oxidative quality in order to produce ATP required for oxidative muscle contraction of the heart.

The cumulative results of these investigations have further supported the potential of mitochondrial oxidative capacity as a therapeutic target in cardiac myopathies. Specifically, we have extended upon previous evidence by demonstrating a specific area of cardiac mitochondrial characteristics responsible for poor metabolism during heart disease. We present novel evidence that the obesity paradox that is seen during aged obesity-induced cardiac hypertrophy could be counteracted by mitigating detrimental mt-mRNA translation initiation to improve functional characteristics (1, 15). Furthermore, we are the first to present evidence of mitochondrial oxidative alterations in the unique form cancer-induced cardiac atrophy – a more prevalent form of heart disease as efficacy of cancer treatments are improving (16). Many avenues of targeting mitochondrial therapeutics to treat cardiovascular disease have been anticipated but few have focused on the formation of mtDNA-encoded proteins in the development of ETC complexes for OXPHOS efficiency. A potential deterioration in the abundance of OXPHOS proteins encoded by mtDNA relative to total volume of the mitochondria has not been explored during heart disease.

The relative contribution of mitochondrial content to total mass of contractile tissue in striated muscle is dysregulated during metabolic disease states such as insulin resistance or obesity in both skeletal and cardiac muscles (8–10, 12). Here, we present with markers of mitochondrial content in the heart indicating this condition is similar within the heart during various disease states. By utilizing multiple disease models with a common downregulation of

mtIF2 and genetic approaches to reduce expression of mtIF2 in cardiac musculature, we demonstrate that reduction in mt-mRNA translation are seen concomitant with an increase in markers of mitochondrial content. This suggests that an elevation in total mitochondrial content with insufficient OXPHOS machinery to match metabolic demand. It stands to reason that a reduction in mt-mRNA translation results in fewer ETC complexes and a reduction in the $\Delta\psi$ of the mitochondria limiting ATP production at complex V. Though not yet supported by experimental evidence, it is possible that a reduction in ATP production of the cardiac mitochondrial pool causes a reciprocal promotion of mitochondrial network expansion to cope with oxidative stress (5). This would mean an increase in mitochondrial content *as a result of poor oxidative phosphorylation* in the pool of mitochondria at the onset of disease provides a means to restore energy balance -- referred to in the literature as mitochondrial retrograde signaling (5). Of course, this is contrary to traditional scientific thought wherein an increase in mitochondrial content is a result of insufficient mitochondrial fusion and degradation of poorly function components (9). Our results support mitochondrial turnover as a causal proponent of greater mitochondrial content in cancer-induced cardiac metabolic perturbations. However, the former conclusion could be supported by our results in hypertrophic cardiomyopathy and genetically altered cardiomyocytes though further experiments would be needed to confirm mitophagy is not a factor.

In conclusion, the research investigations presented within this dissertation are the first to establish mitochondrial mRNA translation as a process that is dysregulated during cardiometablic disease and as a potential therapeutic target to enhance oxidative characteristics of the myocardium. mtIF2 presents as a key regulator for the process of mt-mRNA translation and is necessary for maintain oxidative capacity in cardiac muscle. A disruption in the

expression of mtDNA has deleterious effects on the assembly of OXPHOS complexes; specifically complexes I and IV based on the number of core subunits encoded by mtDNA. Alterations in the flow of electrons through these specific complexes has strong implications for the production of ROS and the subsequent consequences on mtDNA integrity. Any metabolic alterations that result in mtDNA mutations can propagate to further detriments in OXPHOS complex assembly leading to further ROS production and even greater mtDNA mutations causing a 'downward spiral' of metabolic disturbances (23). This series of events has the potential for altering the future care of many cardiac disease states including cardiac hypertrophy, coronary artery disease, and age-induced congestive heart failure.

Future Directions

This series of experiments has taken clear steps to improve our knowledge in a series of cardiomyopathies; however, it leaves open many future lines of research to build upon these findings. Primary experiments that could be used with these experimental models would be the quantification of ROS production and mtDNA mutations and mutation rates. These experiments would directly connect our findings to a prolonged propensity for deleterious effects on the heart. Furthermore, these experiments have tested the necessity of mtIF2 in maintaining cardiac oxidative capacity -- it is yet to be seen if mtIF2 is sufficient to enhance hypoxic resistance in adult cardiac muscle. These future experiments would clearly pave the way for translational experiments to use mtIF2 exogenous expression to mitigate deleterious effects of cardiomyopathies. If this is extended to a mitigation of mtDNA mutation through reduction in ROS production, it would pave the way for age mitigating therapeutic approaches.

Another area that requires future research is in characterization and mitigation of cardiotoxic effects of therapeutic pharmaceutical interventions already implemented in a clinical

setting (16). While multiple approaches assist in treating diseases associated with aging and obesity (Metformin)(19) and cancer (Doxorubicin, Nivolumab)(17), the side-effects are often deleterious to cardiac musculature. Clinical interventions are improving that have greatly increased survival rates of metabolic diseases and cancers, but these are narrow minded and counterintuitive if the resulting outcome is an imminent, fatal cardiovascular event. Researchers need to clearly identify the adverse outcomes on the myocardium of interventions to treat cancer (radiation, chemotherapy, immunocheckpoint blockade) and other age-related disorders (hormone therapies, metabolic therapy, corticosteroid therapy, anti-dementia medications). The side-effects of these treatments have clear negative side-effects on the myocardium and could be contributors to greater mortality. Along these lines, a key initiative that should be the focus of future research in medical administration and organization is to identify the concern of primary care providers who administer these medications and if they are considerate of the complicating effects on patients with or predisposed to cardiovascular disease. A wonderful example of the positive outcomes possible is the rapidly progressing field of cardio-oncolgy wherein specialists combine expertise to identify optimal treatment approaches to enhance outcomes of both oncologists and cardiologists.

Limitations

Various limitations should be taken into account when attempting to generalize the results presented here. A primary limitation of concern is the lack of *in vivo* functional cardiac information including left ventricular ejection fraction, left ventricular internal diameter measures and ventricular wall dimensions. These are all primary markers of cardiovascular disease that can only be measured in a living animal and were not collected in either of the animal studies presented here.

For many of the findings taken from mouse diseases models, a primary concern in applying this to a broader application is the drastically different hemodynamic and microfluidic aspects of small animal cardiovascular system. In fact, when comparing characteristics of the heart across dozens of mammalian species, a relationship between body weight, heart mass, and heart rate is readily apparent (18). A smaller mammal such as a mouse may maintain a heart rate over 800bpm while a fully grown Asian elephant's average heart rate is ~35bpm (2). Much of the differences relate to Frank-Startling contractile forces and outflow capacity through the aortic vasculature (6). This significant difference in the rate of contraction can result in differences in the metabolic characteristics of the myocardium across species. Similarly, the use of rat cardiomyocytes for cell culture experiments must take into account the progenitor phenotype (more glycolytic, less adult myosin content) compared to adult primary cardiac muscle cells (13, 22).

The use of TPEF to analyze redox species within tissue *post mortum* could be subject to changes caused by oxidation that occurs during sample processing though previous research suggests this is unlikely to alter data interpretation (24). There may also be some overlap in the endogenous fluorescence signal of NADH and collagen SHG potentially confounding these results. Likewise, NADPH and NADH species are indistinguishable using this endogenous fluorescence approach. While NADPH may only make us ~5% of the total pool of NADPH/NADH in a cell, it is assumed that this is included in our total measurements of NADH (20).

Limitations that apply to the biochemical analyses must be taken into account as well. For the Seahorse Bioenergetics analysis, it must be assumed that the combination of Antimycin and Rotenone (combinations of Rotenone A, B and C) are sufficient to entirely block electron

flow through OXPHOS complexes though this has not been directly, experimentally confirmed. We were unable to determine production of ROS through MitoSOX analysis in sh-mtIF2 experiments because of overlapping fluorescent signal with the mCherry-tagged plasmid. Additionally, measurements of mtDNA content and mtDNA mutation rates were not performed nor were activity measurements of the various OXPHOS complexes but only the content via immunoblot analysis. Finally, the MTT assay requires oxygen to generate formazan and is subject to alterations from ROS. While commonly used in the literature, this could be confounded by the hypoxia/reoxygenation required from the experimental protocols. While we attempted to only assess effects of hypoxia on the cardiomyocytes, the cells must be returned to normoxia (and thus reoxygenated) in order to perform MTT assay, the reoxygenation and ROS effects must be noted.

Delimitations

For the experiments presented here, we have decided to forego certain experiments that could be reasonably incorporated but have done so for specific reasons. We have attempted to characterize our mouse models of heart disease by highlighting alterations that are consistent with cardiomyopathy (HIF-1α, PGC-1α, fibrosis, altered oxidative metabolism) in an effort to demonstrate a diseased state even without function measurements of cardiac characteristics (echocardiographic assessment). While we could support these findings by using measures of glycolytic metabolism (immunoblot analysis, activity assays, glycolytic bioenergetics flux analysis), we have not performed these experiments because we feel that we can adequately characterize the stated heart disease with the experiments already performed. Similarly, the *in vitro* experiments have highlighted alterations in metabolic characteristics of cardiomyocytes rather than mature myotubes. This was done in order to mitigate any effects of mitochondrial

metabolism that contribute to muscle cell differentiation and because a primary characteristic of heart disease is a transition towards this embryonic cardiac cell phenotype (14). Additionally, while overexpression of the key mt-mRNA translation factors could improve the application of these findings in a diseased state, we have elected to simply test the necessity of mtIF2 for mitochondrial oxidative capacity. This was because prior to attempting to solve issues with deficient mt-mRNA translation during cardiac disease, we sought to determine if this translation initiation factor is required to maintain characteristics of healthy myocardium. Only then would the importance of extrinsically enhancing the expression of mtIF2 become apparent. While many more experimental approaches could be used to extend these results, we are confident that the findings presented here are sufficient for interpretation of the results as discussed.

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Appendices

1. Protocols

H9c2 culture

H9c2 rat ventricular cardiomyocytes were purchased through ATCC (CRL-1446) and cultured in DMEM containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cells were incubated at 37° and 5% CO_2 with media was changed every second day. Cells were grown until ~80% confluence and subcultured at desired densities for subsequent experimental procedures.

Bioenergetics flux analysis

For cell bioenergetics flux analysis, a Seahorse XFp extracellular flux analyzer (Agilent, Santa Clara, CA) was used. Drug cartridges were preincubated with Seahorse Assay media 24 hours prior to experimenst at 37°C in a non-CO_2 incubator. 200µL of PBS was inserted into the cartridge moats to allow adequate humidification. Cells were cultured and plated in Seahorse cell plates at $2x10^4$ cells/ well in 200µL of culture media. 200µL of PBS added to the cell plate moats to allow adequate humidification. On the day of the experiment, Seahorse assay medium was made using Seahorse XF base medium and adding 7mM glucose, 2mM sodium pyruvate, and 2mM L-glutamine then pH was adjusted to 7.36-7.40. Using this complete assay media, drugs were reconstituted as follows: 252µL in oligomycin drug vial for a final concentration of 50µM, 288µL in FCCP drug vial for a final concentration of 50µM, and 216µL in Rotenone/antimycinA vial for a final concentration of 25µM. From these stock drugs, 20µL of oligomycin was added to port A of the drug cartridge (1µM final concentration), 22µL of FCCP was added to port B of drug cartridge (1µM final concentration), and 25µL rotenone/antimycinA was added to port C (0.5µM final concentration. This prepared drug cartridge was loaded into the Seahorse XFp

analyzer at the start of the experiment as instructed by the machine's GUI. The culture media for the cells was removed and rinsed thrice with complete Seahorse assay media and left at a final volume of 180µL/well to allow dilution to desired concentrations from drug cartridge. ~15 minutes after inserting the drug cartridge into analyzer, the hydrating plate can be removed and the cell plate inserted according to instructions given by the machine's GUI. Analysis will be performed over 100 minutes and the drug cartridge and cell plate can be removed and used for further analyses if desired.

Cell Hypoxic exposure and MTT Assay

$2x10^4$ cells/well are plated in a 96-well plate and incubated with culture media for 24 hours at 20% O_2 and 5% CO_2. After desired experimental intervention (transfection, drug incubation), media was replaced with media deplete of serum and cells were placed in a dual gas controlled incubator sub-chamber (Oxycycler C42, Biosperix, Parish, NY). Oxygen was flushed by nitrogen and maintained at 1% O_2 and 5% CO_2 for 6 hours. Following the hypoxic exposure, 1mM MTT (M6494, Invitrogen) was added to cells and incubated at ~20% O_2 for 2 hours. The resulting formazan crystals were solubilized by addition of 100µL af 350mM SDS in 0.01% HCl on gentle shaking for 2 hours. Absorbance was read at 570nm and compared to absorbance of a control plate incubated in normoxic conditions.

Two photon excitation fluorescence

Two-photon excitation fluorescence (TPEF) was used to measure endogenous fluorescence of NADH, FAD, and the second harmonic generation signal of collagen (SHG). Laser excitation by MaiTai Ti:Sapphire laser source (Spectra-Physics, Santa Clara CA) was tuned to 755nm (NADH), 800nm (SHG), or 860nm (FAD). Images were acquired using a resonant-galvo scanner and GaAsP photomultipler tubes (H7422-40, Hamamatsu) with

460/40nm (NADH), 525/45nm (FAD), 400/40nm (SHG) and 600/70nm (Rhodamine) filters. 10μm section of the left ventricle were cut along the short axis. Slides were imaged (512x512 pixels, 16bit depth, 260μm^2). The pixel-wise FAD/(NADH+FAD) ratio normalized to rhodamine was calculated to determine the optical oxidation/reduction ratio. Multiple fields were imaged for each sample and averaged to represent an individual biological sample. Image analysis was completed using MATLAB (MathWorks, Natick, MA).

2. MATLAB Editor Code

Redox ratio calculations in whole tissue microscopy

```
%Create a nice image (avgi2) by combining the FAD and NADH
%channels


% Select your highest level folder containing all the data
start_path = uigetdir('Select a folder');
cd(start_path)


% Creates a list of all NADH and FAD folders
list_755 = dir('*750*');
list_860 = dir('*860*');
list_800 = dir('*800*');



% Goes into each folder, takes the Chan B or Chan C image depending on the
% wavelength.
for i = 1:length(list_755)
redox_755 =
imread(strcat(start_path,'/',list_755(i).name,'/','ChanB_0001_0001_0001_0001.tif'));
avg_755{1,i} = mean(mean(redox_755));
redox_755=double(redox_755);
redox_images{1,i} = redox_755;
```

```
end
for i = 1:length(list_860)
redox_860 =
imread(strcat(start_path,'/',list_860(i).name,'/','ChanC_0001_0001_0001_0001.tif'));
avg_860{1,i} = mean(mean(redox_860));
redox_860=double(redox_860);
redox_images{2,i} = redox_860;
end
for i = 1:length(list_800)
redox_800 =
imread(strcat(start_path,'/',list_800(i).name,'/','ChanA_0001_0001_0001_0001.tif'));
avg_800{1,i} = mean(mean(redox_800));
redox_800=double(redox_800);
redox_images{3,i} = redox_800;
end

%Calculate redox images
for i = 1:length(list_755)
redox_images{4,i} = redox_images{2,i}./(redox_images{2,i}+redox_images{1,i});
avg_redox{1,i} = mean(mean(redox_images{4,i}));
end

%save('AverageRedoxImage',imavgredox);
```

```
mf=max([avg_860{1,:}]);

mn=max([avg_755{1,:}]);


%smooth images and index redox_images structure using same color

%limits for all images

for i=1:length(list_755)

h=fspecial('gaussian',[2 2],5/3);

FADfilt=imfilter(redox_images{2,i},h);

NADHfilt=imfilter(redox_images{1,i},h);

redox = redox_images{4,i};


avgi=(mean(mean(redox_images{2,i}))+mean(mean(redox_images{1,i})))/2;


%Keep this consistent for all your images. Lowering uplim will make it

%brighter

uplim=1000;%adjust these based on your image intensity values

botlim=400;%adjust until you see the pixels gone from background


avgi2=(avgi-botlim)/(uplim-botlim);

avgi2=avgi2.*(avgi2<1)+(avgi2>=1);

avgi2=avgi2.*(avgi2>=0);


%reassign redox ratio jet colors to a range of your choosing based on uplim
```

```
%and botlim, then discretize the redox values from 1-64.
cmj=jet;

%DO NOT CHANGE THESE VALUES
uplim=1.0;
botlim=0.4;

redoxfn=(redox-botlim)/(uplim-botlim);
redoxfn=redoxfn.*(redoxfn<1)+(redoxfn>=1);
redoxfn=round(63*(redoxfn.*(redoxfn>=0)))+1;

uplim=750;
botlim=400;

SHGfn = (redox_images{3,i}-botlim)/(uplim-botlim);
SHGfn = SHGfn.*(SHGfn<1)+(SHGfn>=1);
SHGfn = round(63*(SHGfn.*(SHGfn>=0)))+1;

%assemble the redox ratio map image in increments of different redox ratio values
RYR24=zeros(size(redoxfn,1),size(redoxfn,2),3);

for j=1:64
imm=(redoxfn==j);
```

```
RYR24(:,:,1)=RYR24(:,:,1)+cmj(j,1).*imm.*avgi2;
RYR24(:,:,2)=RYR24(:,:,2)+cmj(j,2).*imm.*avgi2;
RYR24(:,:,3)=RYR24(:,:,3)+cmj(j,3).*imm.*avgi2;
end
redox_images{5,i} = RYR24;
end
%Save the image structure
save('images_matrices.mat','redox_images')

%Export matrix of values to excel with row1-4(NADH, 2HG, FAD, redox) and
%row as the transposed averaged for each variable
Excel_summary = [avg_755; avg_800; avg_860; avg_redox];
%Excel_summary{5,1} = mean([avg_755{1,:}]);
%Excel_summary{5,2} = mean([avg_800{1,:}]);
%Excel_summary{5,3} = mean([avg_860{1,:}]);
%Excel_summary{5,4} = mean([avg_redox{1,:}]);
excel_summary = cell2mat(Excel_summary);
xlswrite('excel_summary',excel_summary);

%Display redox image and filtered FAD and NADH images side by side
figure;imagesc(redox_images{3,1}); colormap(gray);
%subplot (1,3,1), imagesc(redox_images{5,2});colormap(jet); axis square; title 'redox';
%subplot (1,3,2), imagesc(redox_images{2,2});colormap(gray); axis square; title 'FAD';
```

```
%subplot (1,3,3), imagesc(redox_images{1,2});colormap(gray); axis square; title 'NADH';
```

Redox ratio analysis in culture cells

```
% Select your highest level folder containing all the data
start_path = uigetdir('Select a folder');
cd(start_path)

% Creates a list of all NADH and FAD folders
list_755 = dir('*750*');
list_860 = dir('*860*');

% Parse out the folder names so data can be divided up correctly. This code
% segment looks for the first part of the filename before the 755 or 860
% in the filename

celllabel = cell(1,length(list_755));
for i = 1:length(list_755)
underscore_id(i,:) = strfind(list_755(i).name,'_');
celllabel{i} = list_755(i).name(1:underscore_id(i,2)-1);
end
clabel = unique(celllabel);
```

```
% Goes into each folder, takes the Chan B or Chan C image depending on the
% wavelength. Based on the 755 folder, creates 7 ROIs - the first one
% always corresponds to the background and the next six selections are
% cells. Repeat this for all folders. Once you complete 1 folder, the image
% for the next folder pops up immediately.
roi_755 = cell(1,length(list_755));
roi_860 = cell(1,length(list_860));
for i = 1:length(list_755)
redox_755 =
imread(strcat(start_path,'/',list_755(i).name,'/','ChanB_0001_0001_0001_0001.tif'));
roi_755{1,i} = kmultiroi(redox_755,3);
redox_860 =
imread(strcat(start_path,'/',list_860(i).name,'/','ChanC_0001_0001_0001_0001.tif'));
roi_860{1,i} = kroistack(redox_860, roi_755{1,i});
close all
end
save 'analyzed_images.mat' roi_755 roi_860
%%
% Based on the initial filename groups we created, collects mean
% fluorescence values from all plates that belong to the same experimental
% group.
k = 1;
c = 1;
```

```
redox_values = struct('mean_755',[],'mean_860',[]);
for i1 = 1:length(list_755)
%if (~strcmp(list_755(i1).name(1:underscore_id(i1,2)-1),clabel{k}))
%   k = k+1;
%   c = 1;
%   redox_values(k).mean_755 = [];
%   redox_values(k).mean_860 = [];
%end
redox_values.mean_755 = cat(2,redox_values.mean_755,roi_755{i1}.mean(2:6)-
roi_755{i1}.mean(1));
redox_values.mean_860 = cat(2,redox_values.mean_860,roi_860{i1}.mean(2:6)-
roi_860{i1}.mean(1));
redox_values(c).cellplate = celllabel{i1};
redox_755 =
imread(strcat(start_path,'/',list_755(i1).name,'/','ChanB_0001_0001_0001_0001.tif'));
redox_values.img_755{c} = redox_755;
redox_860 =
imread(strcat(start_path,'/',list_860(i1).name,'/','ChanC_0001_0001_0001_0001.tif'));
redox_values.img_860{c} = redox_860;
c = c  + 1;
end
%%
for k = 1:length(clabel)
```

```
redox_values(k).redox_ratio = redox_values(k).mean_860./(redox_values(k).mean_755 +
redox_values(k).mean_860);
end
save 'analyzed_data.mat' redox_values
```

Redox image generation to display for publications

```
sampleid = 2;
load analyzed_data.mat
NADH = double(redox_values.img_755{1, sampleid});
FAD = double(redox_values.img_860{1, sampleid});
redox = FAD./(FAD+NADH);
mf=max(FAD(:));
mn=max(NADH(:));

%h=fspecial('gaussian',[1 1],5/3);
%FADfilt=imfilter(FAD,h);
%NADHfilt=imfilter(NADH,h);
%redox = imfilter(redox,h);

avgi=(FAD+NADH)/2;

%Keep this consistent for all your images. Lowering uplim will make it
```

```
%brighter
uplim=100;%adjust these based on your image intensity values
botlim=20;%adjust until you see the pixels gone from background

avgi2=(avgi-botlim)/(uplim-botlim);
avgi2=avgi2.*(avgi2<1)+(avgi2>=1);
avgi2=avgi2.*(avgi2>=0);

%reassign redox ratio jet colors to a range of your choosing based on uplim
%and botlim, then discretize the redox values from 1-64.
cmj=jet;

%DO NOT CHANGE THESE VALUES
uplim=1;
botlim=0;

redoxfn=(redox-botlim)/(uplim-botlim);
redoxfn=redoxfn.*(redoxfn<1)+(redoxfn>=1);
redoxfn=round(63*(redoxfn.*(redoxfn>=0)))+1;

%assemble the redox ratio map image in increments of different redox ratio values
RYR24=zeros(size(redoxfn,1),size(redoxfn,2),3);
```

```
for j=1:64
imm=(redoxfn==j);
RYR24(:,:,1)=RYR24(:,:,1)+cmj(j,1).*imm.*avgi2;
RYR24(:,:,2)=RYR24(:,:,2)+cmj(j,2).*imm.*avgi2;
RYR24(:,:,3)=RYR24(:,:,3)+cmj(j,3).*imm.*avgi2;
end

%Display redox image and filtered FAD and NADH images side by side
figure;
subplot (1,3,1), imagesc(RYR24); axis square; title 'redox'
subplot (1,3,3), imagesc(NADHfilt);colormap(gray); axis square; title 'NADH'
subplot (1,3,2), imagesc(FADfilt);colormap(gray); axis square; title 'FAD'
```

3. Ethics Committee Approval Letters

UNIVERSITY OF ARKANSAS

Office of Research Compliance

July 09, 2015

MEMORANDUM

TO: Dr. Nicholas Greene

FROM: Dr. Ines Pinto
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 15025

Protocol Title: "Mitochondrial Degeneration in the Onset of Cancer-Cachexia Induced Muscle Atrophy"

Approved Project Period: Start Date: July 9, 2015
Expiration Date: July 8, 2018

The Institutional Biosafety Committee (IBC) has approved Protocol 15025, "Mitochondrial Degeneration in the Onset of Cancer-Cachexia Induced Muscle Atrophy" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

109 MLKG • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572 • Fax: 479-575-6527 • http://rscp.uark.edu

UNIVERSITY OF ARKANSAS | Research & Economic Development
Research Compliance

June 9, 2017

MEMORANDUM

TO: Dr. Nicholas P. Greene

FROM: Ines Pinto, Biosafety Committee Chair

RE: New Protocol

PROTOCOL #: 17034

PROTOCOL TITLE: Cardiac Oxidation Requires Mitochondrial Translation Initiation

APPROVED PROJECT PERIOD: Start Date June 8, 2017 Expiration Date June 7, 2020

The Institutional Biosafety Committee (IBC) has approved Protocol 17034, "Cardiac Oxidation Requires Mitochondrial Translation Initiation". You may begin your study after certification of the biosafety cabinet in HPER 321K.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Nicholas Greene

FROM: Craig N. Coon, Chairman

DATE: 7/13/15

SUBJECT: IACUC Approval

Expiration Date: Jan 1, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol 15065: "Mitochondrial Degeneration in the Onset of Cancer-Cachexia Induced Muscle Atrophy" you may begin work immediately

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Jan 1, 2018 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
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UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Dr. Tyrone Washington

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee (IACUC)

DATE: September 8, 2014

SUBJECT: IACUC APPROVAL
Expiration date: September 4, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED protocol 15004: *'Sarcopenic obesity and skeletal muscle regeneration'*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond September 4, 2017 you must submit a new protocol prior to that date. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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